REMARKS/ARGUMENTS

By the present proposed amendment, four (4) claims are amended and five (5) claims are cancelled. Claims 47, 54-59 and 61 are pending. No fees for claims are believed payable. Applicants submit that no new matter has been added by the present proposed claim amendments and no change in inventorship is believed to result. Entry of the proposed amendments is respectfully requested.

Claim 47 is amended to specify that the immunoglobulin-polypeptide chimera is soluble. Support for this amendment can be found in the specification as filed at least at page 9 line 8 – page 10, line 3. Claim 47 is also amended to specify that the polypeptide has the sequence of SEQ ID No: 4 which corresponds to GAD2. Support for this amendment can be found in the specification as filed at least at page 39, lines 5 – 10.

The presently pending claims stand rejected under 35 U.S.C. § 103(a) as being unpatentable over WO 98/30706 in view of Liu *et al.* (1992) *PNAS* 97(26): 283-292 ("Liu"). According to the Office Action, it would have been obvious to one of ordinary skill in the art, at the time the invention was made, to produce the construct of WO 98/30706 employing the T-cell epitopes of SEQ ID NOs: 3 and 4, as taught by Liu (Office Action at page 2). Applicants respectfully traverse this rejection.

I. No *prima facie* case established.

To establish a *prima facie* case of obviousness under 35 U.S.C. § 103, the Office must first demonstrate that a prior art reference, or references when combined, teach or suggest all claim elements. See, *e.g., KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1740 (2007); *Pharmastem Therapeutics v. Viacell et al.*, 491 F.3d 1342, 1360 (Fed. Cir. 2007); *Abbott Laboratories v. Sandoz, Inc.*, 529 F.Supp. 2d 893 (N.D. Ill. 2007) and MPEP § 2143(A)(1). In addition to demonstrating that all elements were known in the prior art, the Office must also articulate a reason for combining the elements. See, *e.g., KSR* at 1741; *Omegaflex, Inc. v. Parker-Hannifin Corp.*, 243 Fed. Appx. 592, 595-596 (Fed. Cir. 2007) citing *KSR*.

As will be discussed in detail below, Applicants respectfully submit that in the instant case, each and every element of the claims as amended herein are not in the prior art of record and the prior art does not provide a rationale for making the modifications required to arrive at the claimed invention. As such, the asserted *prima facie* case of obviousness fails.

A. Each and Every Element of the Claims Not Disclosed in the Prior Art.

Claim 1 and all claims depending there from as amended herein specify that the composition is a "*soluble* immunoglobulin-polypeptide chimera." The prior art of record is silent as to a soluble immunoglobulin-polypeptide chimera. Because this element is not in the prior art of record, no *prima facie* case of obviousness has been established. Withdrawal of this rejection is respectfully requested.

B. No Rationale to Combine the Prior Art to Arrive at the Claimed Invention.

As set forth above, the mere fact that prior art may be modified to produce the claimed product does not make the modification obvious unless the prior art suggests the desirability of the modification. The prior art of record simply does not teach or suggest the desirability of providing a soluble Ig-GAD2 construct as claimed.

The purported rationale in the Office Action appears to be a reference in Liu to articles stating that p206 and p524 are immunodominant epitopes in T1D. However, the law is clear that prior art must be considered in its entirety, including disclosures that teach away from the claims. MPEP 2141.03 VI. Despite the references by Liu to these other papers, the totality of the Liu references teaches away from the presently claimed invention.

As previously set forth, Liu demonstrated that in the *spontaneous* NOD mice that had not been immunized, "tetramer staining results showed that the tetramers detected T cells infiltrating the islets of NOD mice with a percentage that is not significantly above the background..." Liu at page 14600, Col. 1. Therefore, Liu's results indicate that p206- and p524-reactive T cells are **not** spontaneously present in NOD mice at detectable levels. *Id.* Type 1 diabetes is a spontaneous disease. Based on Liu's results (barely staining for only one peptide in immunized mice and no staining for either peptide in non-immunized mice), one of skill in the art would not have thought the p206 and p524 peptides were involved in activation of autoreactive T cells during development of diabetes. As such, a person of ordinary skill in the art reading Liu **in its entirety** (not merely focusing on a couple sentences referening to other articles suggesting that p206 and p524 are immunodominant in T1D) at the time of Applicants' invention would not have had any motivation to use the p206 or p524 peptides for insertion into the construct of WO 98/30706. Instead, such a person would have been led in a direction divergent from the path that

was taken by the Applicant since the p206 and p524 peptides failed to activate autoreactive T cells in non-immunized NOD mice. Based on Liu's actual results and conclusions, one of skill in the art would not have thought that the peptides of Liu were involved in the onset of T1D.

This is not a situation involving combination of prior art elements to yield predictable results or simple substitution of one known element for another to yield predictable results. In fact, none of the exemplary rationales set forth at MPEP 2143 have been established. Because no rationale exists to combine Liu with WO 98/30706 exists, no *prima facie* case of obviousness has been established. Withdrawal of the instant rejection is respectfully requested.

II. Secondary Indicia of Non-Obviousness.

It is well settled that a *prima facie* case of obviousness is rebuttable by proof that a claimed compound possesses unexpectedly advantageous or superior properties. MPEP 2145 VII. Even assuming, *arguendo*, that a *prima facie* case of obviousness has been established, which is not admitted, Applicants submit herewith a Rule 132 Declaration of co-inventor Dr. Habib Zaghouani (the "Zaghouani Declaration") setting forth several unexpected properties of the presently claimed composition.

A. Soluble But Not Aggregated Chimera Unexpectedly Delayed T1D When Administered After IAA Seroconversion.

WO 98/30706 generally discloses peptide delivery on Igs increases presentation efficiency compared to antigen alone. Because, even prior to filing of the instant application, T1D was suspected to involve multiple autoantigens and the initiating antigen was unknown (See *e.g.* Delovitch attached herewith), it would not have been expected that restoration of normoglycemia, if possible at all, would be attained without modulation of diverse T cell specificities through a combination of capture of T cells (signal 1) and bystander suppression, without activating co-stimulatory signal 2. (See Tisch and Bach attached herewith).

It was known prior to the filing date of the present invention that cross-linking of Fc receptors on target cells by antigen-antibody complexes could trigger the production of anti-inflammatory cytokines such as IL-10 which were known to be important for down regulating T cells engaged to antigen presenting cells as well as neighboring T cells (bystander suppression). (See Deo, Polat and Sutterwala attached herewith). Moreover, aggregation of Igs was known to

confer effector functions associated with the Fc fragment without the need for complex formation. (See Christian and Rosenqvist attached herewith).

In view of the foregoing, at the time the instant application was filed, one of skill in the art would not have expected a *soluble* Ig-peptide chimera to be effective at treating or delaying T1D at least because soluble Ig-peptide chimera would not have been expected to induce Fc receptor cross-linking and subsequent production of cytokines such as IL-10 as would have been thought to be required for bystander suppression. In fact, in two post-filing date papers co-authored by Applicants, it was shown that Ig-GAD1 and IgINSβ induce T regulatory cells and prevent T1D *only* when given in aggregated, but not soluble form. Indeed, this was likely because the aggregated but not soluble Ig-peptide chimeras cross-linked Fcγ receptors on antigen presenting cells and induced IL-10 production by the APCs thereby leading to bystander suppression and expanded T regulatory cells. (See Gregg 2004 and Gregg 2005 attached herewith).

Despite the fact that soluble Ig-GAD2 does not induce cross-linking of Fc receptors, Applicants have surprisingly discovered that it effectively delays T1D when administered after IAA seroconversion (a relatively late stage of disease) and prevents diabetes when given at the prediabetic stage whereas aggregated Ig-GAD2 failed to delay diabetes when given at the insulitis stage. Particularly surprisingly, soluble Ig-GAD2 asserts its effect via a mechanism independent of IL-10-mediated bystander suppression. As disclosed in the Zaghouani Declaration attached herewith, even when cytokine neutralization was performed along with soluble Ig-GAD2 treatment, recovery from diabetes persisted with anti-IL-10 treatment but was nullified by removal of IFNγ. These observations indicate that IFNγ, contrary to its well defined inflammatory function, is actually involved in modulation of inflammation and restoration of normoglycemia. These unexpected findings simply could not have been predicted by the ordinary skilled artisan at the time the instant application was filed.

B. Soluble Ig-GAD2 Therapy Unexpectedly Increases Healthy Islet Cells and Promotes Islet Cell Regeneration.

Even assuming, *arguendo*, one of skill in the art at the time the present application was filed had motivation to combine WO 98/30706 with the peptides of Liu and had a reasonable expectation of inducing tolerance (which is not admitted), such a person would not have had any

expectation that the resultant construct, when prepared in soluble form, would increase the number and health of islet cells when administered after IAA seroconversion.

As set forth in the Zaghouani Declaration attached herewith, while most of the islets in hyperglycemic and diabetic control mice exhibited intra-insulitis, the majority of islets in mice treated with the soluble Ig-GAD2 construct were not inflamed or had only mild peri-insulitis. Furthermore, histopathologic analysis indicated that treated mice had significantly greater number of islets when compared to both hyperglycemic and diabetic mice. Analysis of islet infiltration scores among the different groups of mice indicated that the 15-week soluble Ig-GAD2 treatment group had a higher number of islets with periinsulitis or no insulitis relative to the hyperglycemic stage. On the other hand, the number of islets with severe- and mild-intrainsulitis were reduced in the treated versus hyperglycemic mice. Surprisingly, in the 25-week soluble Ig-GAD2 treatment group, although the total number of islets was reduced to that of the hyperglycemic stage, most of these islets exhibited no, peri- or mild intra-insulitis. Overall, the treatment with soluble Ig-GAD2 led to a significant increase in the number of noninflammed ("healthy") islets that restored and maintained normoglycemia. This result was unexpected.

An experiment was then conducted to determine whether the healthy islets were a result of a regression of inflammation and/or regeneration of beta cells. As set forth in the Zaghouani Declaration, sections from hyperglycemic mice showed very few insulin-producing beta cells and no BrdU incorporation resulting in an insignificant number of $BrdU^{+}/insulin^{+}$ beta cells. By contrast, islets from the 25-week soluble Ig-GAD2 treatment group showed beta cells that stained positive for insulin and were either BrdU negative (previously generated beta cells) or BrdU positive (newly generated beta cells). Notably, the number of these insulin-producing regenerating beta cells was significantly increased in all five soluble Ig-GAD2 treated mice in which treatment restored normoglycemia. Interestingly, the total number of dividing cells producing insulin ($BrdU^{+}/insulin^{+}$) was low and may not solely account for the restoration of normoglycemia. $BrdU^{-}/insulin^{+}$) residual islet cells, which amounted to 81 cells per pancreas, may have also contributed to the control of blood glucose levels, and these likely represent a combination of newly formed and residual beta cells that were rescued by regression of infiltration.

Collectively these findings suggest that soluble Ig-GAD2 therapy reduces islet cell infiltration leading to rescue of residual and formation of new β cells. These surprising and unexpected results could not have been predicted by a person of ordinary skill in the art at the time the instant application was filed. Withdrawal of the instant rejection is respectfully requested.

CONCLUSION

Applicants have provided a novel soluble Ig-GAD2 composition that surprising can reverse diabetes after IAA seroconversion in NOD mice and stimulate regeneration of healthy islet cells. These unexpected findings represent a great and far-reaching advance in the art. Applicants believe the application is in condition for allowance. Early and favorable consideration is respectfully requested.

Respectfully submitted,

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Enclosures

Delovitch et al., The nonobese diabeteic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. Immunology Vol. 7, 727-738 (1997).

Tisch, R et al., 1996. Insulin-dependent diabetes mellitus. Cell. 85:291-297.

Bach, J.F. 1994. Insulin-dependent diabetes mellitus as an autoimmune disease. Endoc. Rev. 15:516-542.

Deo, Y.M. et al., 1997. Clinical significance of IgG Fc receptors and FCγR-directed immunotherapies. Immunol. Today. 18:127-135.

Polat, G.L. et al. 1993. Cross-linking of monocyte plasma membrance Fc alpha, Fc gamma or mannose receptors induces TNF production. Immunology. 80:287-292.

Sutterwala, F.S. et al. 1998. Reversal of proinflammatory responses by ligating the macrophage Fcg receptor type I. J. Exp. Med. 188:217-222.

Christian, C.L. 1960. Studies on aggregated gamma-globulin I & II. J. Immunol. 84:112-121.

Rosenqvist, E. et al., 1987. Thermal properties of human IgG. Mol. Immunol. 24:495-501.

Gregg et. al., 2004. A sudden decline in active membrane-bound TGF-beta impairs both T regulatory cell function and protection against autoimmune diabetes. J. Immunol. 173:7308-7316.

Greg et al., 2005. IL-10 diminishes CTLA-4 expression on islet-resident T cells and sustains their activation rather than tolerance. J. Immunol. 174:662-670.

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The Nonobese Diabetic Mouse as a Model of Autoimmune Diabetes: Immune Dysregulation Gets the NOD

Review

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Who is more contemptible than he who scorns knowledge of himself?—John of Salisbury

This statement reflects our concern about the factors, both internal and external, that control our lives. Unfortunately, there are times when our immune system, which normally protects us from adverse infections and diseases, scorns our body and mounts an autoaggressive attack against it, resulting in autoimmune disease. Among the many severely debilitating autoimmune diseases are multiple sclerosis, rheumatoid arthritis, and insulin-dependent diabetes mellitus (IDDM), or type 1 diabetes. What are the factors, both genetic and environmental (diet and infection) that mediate the onset of these diseases? How do we identify these factors? And how can we control these factors in order to prevent the onset of autoimmune diseases? Studies of autoimmune diseases in representative animal models have proven to be very informative.

This review focuses on the use of one such animal model, the nonobese diabetic (NOD) mouse, which spontaneously develops IDDM. The NOD mouse has become the most extensively studied model of spontaneous organ-specific autoimmune disease. Excellent reviews exist on the origin, genetics, immunological characteristics, and influence of environmental factors on IDDM in NOD mice (Kikutani and Makino, 1992; Leiter and Serreze, 1992). Importantly, experiments conducted with NOD mice in recent years have begun to provide clues about how we may modulate and regulate the immune response in order to protect against IDDM in humans (Bowman et al., 1994; Bach and Mathis, 1997).

The central questions that we address in this review are: What are the immunological mechanism(s) that induce this T cell–mediated autoimmune disease? Is there a single mechanism that elicits IDDM or are there multiple mechanisms? Different mechanisms of IDDM induction have been proposed, including (1) expression of diabetogenic major histocompatibility complex (MHC) class II allelic products that bind peptides with low affinity, enabling self-reactive T cells to escape from the thymus to the periphery; (2) positive selection of specific T cell antigen receptors (TCR) that recognize primary autoantigen(s); (3) breaking of peripheral tolerance by pathogenic infection; and (4) deficient activation of regulatory T cells, resulting in deficient immune regulation.

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The NOD Mouse Model of IDDM

Since the discovery of the NOD mouse about 17 years ago by researchers at the Shinogi Company (Makino et al., 1980), this mouse model has been used to explore the many features of IDDM that are shared with human IDDM (Table 1), including the polygenic control reflected by the inheritance of particular MHC class II alleles and multiple non-MHC loci as genetic risk factors; the transmission of disease by bone marrow–derived hematopoietic stem cells; the early appearance of an intra-islet inflammatory infiltrate (insulitis) and anti-islet cell antibodies; and the autoreactive T cell dependence of IDDM pathogenesis and the ability to intervene with disease progression by modulation of T cell function (Bach, 1994; Bowman et al., 1994; Tisch and McDevitt, 1996).

Considerable evidence suggests that IDDM in NOD mice is mediated by T cells. IDDM is prevented by neonatal thymectomy, by immunosuppressive agents that target T cells, and by anti-CD4 and anti-CD8 monoclonal antibody treatments. Furthermore, IDDM can be adoptively transferred to neonatal NOD mice and immunodeficient NOD.SCID (severe combined immunodeficiency) mice by T cells from spontaneously diabetic adult NOD mice.

IDDM pathogenesis in NOD mice is heralded by the infiltration—first by dendritic cells and macrophages and then by T cells ($CD4^+$ and $CD8^+$) and B cells—of the perivascular duct and peri-islet regions of the pancreatic islets of Langerhans (peri-insulitis) beginning at 3–4 weeks of age. This stage is followed by the slow, progressive, and selective T cell–mediated destruction of insulin-producing islet β cells by 4–6 months of age. Whereas a nondestructive peri-insulitis is observed in all female and male NOD mice, NOD females develop a more invasive and destructive insulitis and incur a higher incidence (80%–90%) of IDDM than males (10%–40%). This pronounced female gender bias is not observed in humans.

A consensus view of the factors eliciting IDDM in the NOD mouse is that dysregulation of the immune response is a principal factor: an excess of islet antigen–specific T helper type 1 (Th1) cells arise, perhaps as a consequence of a deficiency in regulatory or suppressor T cells.

T Cell Responses to Autoantigens in IDDM: Are They Relevant?

Both T cells and B cells reactive to islet antigens infiltrate the pancreatic islets at the inflammatory stage of insulitis. Do these infiltrated B cells play a role in the etiopathogenesis of IDDM? Autoantibodies against several islet autoantigens, such as insulin, glutamic acid decarboxylase 65 (GAD65), and IA-2, a cytoplasmic tyrosine phosphatase, are present in the sera of humans with IDDM for several years before the onset of overt disease, but a direct role for these autoantibodies in the pathogenesis of IDDM remains uncertain. Evidence exists that B cells themselves may play an important role in the etiology of IDDM. B cell–deficient ($Ig\mu^{null}$) (Serreze et

Table 1. Functional T Cell Defects in NOD Mice

T Cell Population	Defect	Associated with	Restored by	References
Splenic T cells	SMLR response	$CD4^+$ T cell defect Reduced IL-2 production	IL-2	Serreze and Leiter, 1988
Thymocytes and peripheral T cells	Response to TCR stimulation	Deficient PKC/Ras/MAPK signaling pathway	IL-2 (partially) IL-4 (completely)	Rapoport et al., 1993a Rapoport et al., 1993b Salojin et al., 1997b
		Reduced IL-2 and IL-4 production	Anti-CD28 monoclonal antibody	Arreaza et al., 1997
Intrathymic and peripheral NK-like T cells	Maturation	Reduced IL-4 production	IL-7	Gombert et al., 1996a Gombert et al., 1996b

MAPK, mitogen-activated protein kinase.

al., 1996) or anti-Igμ–treated female NOD mice (Noorchashm et al., 1997) develop neither insulitis nor IDDM. Although this result has raised some controversy, it is possible that the main role of B cells in the immunopathogenesis of IDDM may not be to secrete autoantibodies but rather to present autoantigens to islet β cell–reactive $CD4^+$ T cells.

Accordingly, attention has been focused in recent years on the role of T cells reactive against islet autoantigens. The identity of islet β cell antigens that may be targets of autoimmune T cells remains controversial, and several different autoantigens have been implicated (Singh et al., 1998; Zechel et al., 1997). Nevertheless, the importance of autoantigens in the etiopathogenesis of IDDM is supported by several lines of study. For example, unlike spleen cells from NOD mice, spleen cells from β cell–depleted NOD mice cannot adoptively transfer IDDM to irradiated NOD mice (Larger et al., 1995), suggesting that β cell autoantigens must be present continuously for development of IDDM. Additional evidence for antigen-driven autoimmunity in IDDM is the loss of self-tolerance in NOD mice upon immunization with antigenic self-peptides (Ridgeway et al., 1996).

Candidate primary autoantigens for the development of IDDM are GAD65 (or the mouse GAD67 isoform), insulin, proinsulin, and heat shock protein 60 (hsp60). T cell responsiveness to GAD65 (Kaufman et al., 1993; Tisch et al., 1993) and insulin (Daniel et al., 1994) is detectable by 3–4 weeks of age in NOD mice, and the induction of neonatal tolerance to GAD65 eliminates the subsequent development of insulitis and IDDM (Kaufman et al., 1993; Tisch et al., 1993; Tian et al., 1996). Treatment of mice with either insulin or insulin B chain (Zhang et al., 1991; Bergerot et al., 1994; Muir et al., 1995), proinsulin (Harrison et al., 1996) or the hsp60 peptide p277 (Cohen, 1997; Elias et al., 1997) also prevents IDDM and downregulates responses to the other three autoantigens, respectively. Of the more than ten known islet autoantigens targeted in IDDM, insulin is the only one expressed specifically by β cells. Nonetheless, insulin, proinsulin, and GAD65 are each unable to elicit IDDM upon active immunization of NOD mice, and only a transient hyperglycemia and insulitis are induced by hsp60 or its p277 peptide (Elias et al., 1995). Thus, at present it is difficult to assign a "primary diabetogenic antigen" status to a single islet autoantigen, consistent with the notion that IDDM in NOD mice and humans is associated with T cell reactivity to many antigen specificities (Roep, 1996).

What Role Do MHC Molecules Play in IDDM?

Do any of the IDDM-susceptibility loci control the function of autoreactive effector and regulatory T cells? The inheritance of particular MHC class II alleles constitutes one of the most important genetic risk factors for susceptibility to IDDM (Wicker et al., 1995; Tisch and McDevitt, 1996; Vyse and Todd, 1996; Wicker, 1997). The unusual $H\text{-}2^g$ MHC haplotype of NOD mice (K^d, $I\text{-}A^g$, $I\text{-}E^{null}$, D^b), which maps in the *Idd-1* susceptibility locus on chromosome 17, contributes to several dysfunctions of antigen-presenting cells (APC) that may promote the development of islet β cell–autoreactive T cells (Atkinson, 1997). The I-Aβ^g allele in NOD mice and certain human leukocyte antigen (HLA)–DQβ alleles in humans that encode serine, alanine, or valine at position 57 mediate IDDM susceptibility, whereas aspartic acid at position 57 is associated with IDDM resistance. Mutation of the I-Aβ^g gene to contain Asp57 reduces the incidence of IDDM but does not prevent insulitis, sialitis, or the development of insulin and nuclear autoantibodies (Lund et al., 1990). This result indicates that alteration of $I\text{-}A^g$ neither prevents the homing of T cells to the pancreas or salivary glands nor eliminates autoreactive T cells, but rather blocks the progression to overt IDDM. When $I\text{-}A^g$ is expressed in the heterozygous state together with various other non-$I\text{-}A^g$ class II molecules in NOD mice, these mice all are resistant to the onset of IDDM (Schmidt et al., 1997). Thus, an IDDM-resistant MHC class II allele on one haplotype may dominate an IDDM-susceptible MHC class II allele on a second haplotype.

The reason that homozygosity at $H\text{-}2^g$ is necessary but not sufficient for the development of IDDM may be that $I\text{-}A^g$ MHC class II molecules are unable to present antigen efficiently to β cell antigen–autoreactive T cells. That is, deletion of self-reactive T cell clones may require a threshold of peptide–MHC complexes that persist on APCs for a finite period of time (Sprent et al., 1988; Milich et al., 1989; Ashton-Rickhardt and Tonegawa, 1994). It is possible that the threshold and time of peptide–MHC expression on APCs in the thymus of an NOD mouse are not sufficient for negative selection of autoreactive T cells by induction of their apoptosis (Serreze, 1993). Indeed, alterations in the differentiation and function of APCs in NOD mice have been reported (Serreze et al., 1993a, 1993b).

That the process of negative selection or inactivation of islet-specific T cells may be deficient in NOD mice is

supported by the recent report that I-A^{g7} MHC class II molecules bind peptides of an islet autoantigen with only low affinity, preventing efficient presentation by NOD APCs to autoreactive T cells (Carrasco-Marin et al., 1996). In a NOD thymus, inefficient MHC class II–mediated autoantigen presentation by APCs in the thymus could result in failure to delete potentially autoreactive T cells, which then exit the thymus and induce autoimmune IDDM. Indeed, T cells from NOD mice have been found to display an abnormally high reactivity to self-proteins (Ridgeway et al., 1996). In addition, it appears that more autoreactive T cells escape from the thymus in NOD mice than in mice resistant to autoimmune disease. These observations provide additional support for the earlier findings that autoreactive T cells are able to activate low-level effector T cell responses in NOD mice (Haskins et al., 1989) and that relatively few peptides can be eluted from I-A^{g7} molecules (Reich et al., 1994).

Nevertheless, support for a central role of I-A^{g7} in the generation of pathogenic T cells is not universal. I-A^{g7} molecules have not been found deficient in either the binding or the presentation of certain autoantigen peptides (Harrison et al., 1997; Reizis et al., 1997; Zechel et al., 1997). In addition, it has been difficult to detect T cell autoreactivity to Islet autoantigens in naive, prediabetic NOD mice. Nevertheless, I-A^{g7} in mice (Carrasco-Marin et al., 1996, 1997) and HLA-DQ3.2 in humans (Buckner et al., 1996) appear to bind most peptides with low avidity, as determined by decreased stability of their peptide–MHC complexes upon denaturation in SDS. In T cell assays, immunogenic peptides, including those from diabetogenic autoantigens, appear to have a fast rate of dissociation from I-A^{g7} molecules, in that the peptide-pulsed NOD APCs are easily washed free of peptide.

Despite the weak binding of I-A^{g7} molecules, it is clear that NOD APCs can still present peptides to reactive T cells. It is possible that other accessory molecules compensate for the low peptide-binding affinity of I-A^{g7}. For example, non–MHC-linked genes encoding costimulatory molecules may affect the activation threshold of T cells in either the thymus or the periphery, and the expression of such costimulatory molecules may be altered in response to low-affinity peptide–MHC complexes. Indeed, impaired expression of costimulatory molecules (CD28) or their ligands (B7-1 and B7-2) on T cells and APCs underlies abnormal T cell activation and anergy in several autoimmune diseases in mice and humans (Tivol et al., 1996; Salojin et al., 1997a), including IDDM. Interestingly, it has been suggested that up-regulation of B7-1 expression activates Th1 cells predominantly, while generation of Th2 cells is more dependent on the CD28/B7-2 pathway. This scenario indicates that generation of Th1 versus Th2 cells is influenced by limiting CD28-B7 costimulation.

Does Molecular Mimicry between Viruses and Islet Autoantigens Break Self-Tolerance and Elicit IDDM?

Molecular mimicry has received considerable attention in recent years in attempts to explain the activation and expansion of autoreactive T cells in the periphery. Molecular mimicry, defined by three-dimensional structural homology, is postulated to exist for T cell epitopes on a viral protein and autoantigen(s). It is thought that most T cell epitopes of an autoantigen are not available for recognition by T cells in the thymus, enabling autoreactive T cells to escape thymic tolerance and to exist in the peripheral T cell pool of healthy individuals and animals (Weckerle et al., 1996). Autoantigenic epitopes might not be recognized in the thymus, either because they are not recognized by T cells (cryptic epitopes) or because they are not generated in sufficient amounts to bind to MHC molecules (Sercarz et al., 1993). T cells specific for these cryptic epitopes may suddenly become evident as a result of a pathogenic viral infection. Accordingly, by molecular mimicry, T cell reactivity to an infectious viral antigen may result in activation and expansion of T cells cross-reactive with an autoantigen.

Is there evidence for molecular mimicry in T cell–mediated autoimmune disease? It is known that experimental acute and persistent infections with DNA or RNA viruses can induce, accelerate, or enhance autoimmune responses and cause autoimmune disease (Oldstone, 1988). In fact, molecular mimicry has been shown to exist between T cell epitopes of a viral protein and an autoantigen in multiple sclerosis, namely myelin basic protein. Evidence in support of the molecular mimicry hypothesis for IDDM (Oldstone, 1997) is that infections with Coxsackie B4 and rubella viruses have each been linked to the induction of IDDM (Forrest et al., 1971; Gamble et al., 1973; Notkins et al., 1984). Interestingly, one of the epitopes of the GAD65 islet cell autoantigen has apparent structural homology to an epitope of the Coxsackie B4 virus (Tian et al., 1994). Moreover, a T cell proliferative response shared between GAD65 and Coxsackie B4 was found among 25% of 16 patients with a new diagnosis of IDDM but among none of 13 healthy matched control subjects (Atkinson et al., 1994). Nonetheless, reciprocal cross-reactivity between anti-GAD65 antibodies and anti-Coxsackie B4 antibodies with Coxsackie B4 and GAD65, respectively, which would support the molecular mimicry hypothesis, has not yet been found. The role of enteroviruses in the etiology of IDDM is presently inconclusive (Graves et al., 1997).

Thus, an immune response elicited against an infecting pathogen may cross-react with self-antigens that share determinants with that pathogen so that an autoimmune disease may be initiated or aggravated by viral infections. Persistent viral infection may explain why T cells infiltrate not only the pancreas in NOD mice but also several other glands (the submandibular salivary glands, thyroid, lachrymal glands, ovary, and testes), indicating that NOD mice exhibit many T cell–mediated inflammatory responses (Bach, 1994). However, it should be kept in mind that infectious agents do not invariably increase the incidence of IDDM, since the incidence of IDDM is highest in NOD mouse colonies housed in specific pathogen–free facilities. Some viral infections even prevent IDDM (von Herrath and Oldstone, 1996).

Regarding specific pathogens and their potential role in the cause of IDDM, superantigens have been implicated in human IDDM (Conrad et al., 1994). More recently, a new endogenous retroviral genome was implicated in the pathogenesis of human IDDM (Conrad et al., 1997). An envelope protein of this retrovirus encodes a superantigen that preferentially expands the Vβ7$^+$ T

cell subset in IDDM patients. This protein has homology with the mouse mammary tumor virus–derived endogenous retroviral superantigen. Thus, this endogeneous superantigen provides evidence for the involvement of a pancreatic islet cell membrane-bound superantigen as a candidate autoimmune gene in IDDM. It has been postulated that the endogeneous retroviral genome is transcribed in lymphocytes and that the superantigen may activate T cells in association with class II MHC molecules. The role of endogeneous retroviral transcripts in the islet cells has also been suggested in IDDM in NOD mice (Gaskins et al. 1992).

Is the Onset of IDDM Mediated by T Cell Clones That Possess a Restricted T Cell Repertoire?

The events that initiate IDDM are not well understood. It is possible that the association between MHC class II molecules and IDDM susceptibility in NOD mice may be due to the recognition of a single MHC class II–restricted antigenic determinant by a clone of autoreactive T cells. Such recognition could lead to an autoimmune response and promote the onset of IDDM, consistent with the notion that several autoimmune disorders may be linked to the restricted use of TCR gene segments (Nepom and Erlich, 1991). In this case, a restricted T cell repertoire targeted to a major autoantigen may represent an important early event in the onset of IDDM. This hypothesis is supported by animal models of experimentally induced autoimmune diseases, such as experimental autoimmune encephalomyelitis, in which immunization with determinants of an autoantigen (myelin basic protein) leads to monoclonal T cell responses that initiate inflammatory responses and ensuing disease. It has proven challenging to develop a test of this hypothesis in an animal model of an autoimmune disease that develops spontaneously, such as IDDM in NOD mice.

Many investigators have isolated islet antigen–specific T cell clones from the periphery as well as lesions of insulitis from both prediabetic and diabetic NOD mice and have shown that these T cells express many different TCRs (Haskins et al., 1988, 1989; Maeda et al., 1991; Nakano et al., 1991; Zipris et al., 1991a; Waters et al., 1992; Toyoda et al., 1992; Galley and Danska, 1995; Daniel and Wegmann, 1996; Komagata et al., 1996; Fox and Danska, 1997). These results have suggested that such islet-specific T cells recognize several different antigens. A more rigorous test of whether a restricted T cell repertoire initiates IDDM requires extensive analysis of TCR expression by islet-infiltrating T cells in very young IDDM-prone NOD mice, at the onset of insulitis at 1 month of age and even prior to insulitis. As previously demonstrated, islet-infiltrating $CD4^+$ T cells examined at the time of insulitis express a heterogeneous array of TCR variable (V) β gene products (Maeda et al., 1991; Nakano et al., 1991; Zipris et al., 1991a; Toyoda et al., 1992; Waters et al., 1992; Galley and Danska, 1995). Because of T cell recruitment, inflammation, determinant spreading of the autoimmune response, or a combination of these, it has been proposed that a TCR-restricted monoclonal population of islet-infiltrated T cells that recognize a single β cell autoantigen initiates islet infiltration, but because of very low frequency may escape detection at the time of insulitis and subsequently during progression to IDDM (Yang et al., 1996). This problem necessitated analyses of TCR expression by T cells at time points preceding histologically detectable infiltration. Assay of the TCR Vβ repertoire of islet-infiltrating T cells in very young NOD mice revealed that one monoclonal TCR Vβ8.2 gene product is expressed by T cells infiltrating the islets of these mice at 2 weeks of age (Yang et al., 1996). The resultant inflammatory response rapidly obscures the monoclonal nature of the initiating event. These findings suggest that IDDM in NOD mice may be initiated by the recognition of a single autoantigen.

More recently, it has also been shown that the majority of T cell clones isolated from spontaneous islet lesions of prediabetic female NOD mice of 4–12 weeks of age are diabetogenic when adoptively transferred and react with a single autoantigen peptide consisting of residues 9–23 of the insulin B chain (Simone et al., 1997). This subset of insulin B9–23–reactive T cell clones expresses no detectable TCR Vβ restriction but is restricted to the expression of a single TCR Vα13.3 chain combined with the Jα45 or Jα34 segments. These data suggest that immunodominant insulin B chain peptides recognized predominantly by restricted TCR Vα chains may play a major role in progression to IDDM in NOD mice, in support of the efficacy of preventive insulin B chain therapy of NOD IDDM (Zhang et al., 1991; Bergerot et al., 1994; Muir et al., 1995; Daniel and Wegmann, 1996; Bergerot et al., 1997). Similarly, islet-infiltrated MHC class I–restricted $CD8^+$ T cell clones obtained from diabetic NOD mice use strikingly homologous TCR Vα and Vβ gene sequences (Santamaria et al., 1995), suggesting that infiltrated $CD8^+$ T cells may also recognize a more restricted set of β cell autoantigen epitopes even after the onset of IDDM. Thus, a limited expression of TCR Vα and Vβ genes by certain subsets of pathogenic T cells may be associated with IDDM onset, but a consensus awaits further experimentation on a much larger number of islet-infiltrating (early and late) T cell clones in both prediabetic and diabetic NOD mice.

Does an Imbalance Between Th1 and Th2 Cell Activation Arise in NOD Mice, and Is This Imbalance Crucial in Determining Whether Autoimmune T Cell Reactivity Results in IDDM?

Considerable evidence indicates that cooperation between $CD4^+$ and $CD8^+$ T cells is required to promote development of IDDM in NOD mice (Bendelac et al., 1987; Christianson et al., 1993) and that islet β cell destruction is mediated by both $CD4^+$ (Haskins and McDuffie, 1990; Christianson et al., 1993; Rohane et al., 1995) and $CD8^+$ (Wicker et al., 1995; Kay et al., 1996; Serreze et al., 1996; Wang et al., 1996; Wong et al., 1996) T cells. Included among the effector cells of IDDM in NOD mice are $CD4^+$ Th1 cells, which preferentially secrete interferon-γ (IFN-γ) and tumor necrosis factor-α (TNFα) (Rabinovitch, 1994; Katz et al., 1995; Liblau et al., 1995; Pilstrom et al., 1995; Trembleau et al., 1995; Shimada et al., 1996; Elias et al., 1997; von Herrath and Oldstone, 1997). Current evidence, albeit indirect, suggests that these effector Th1 cells and susceptibility

to IDDM may be regulated by $CD4^+$ Th2 cells, which preferentially secrete interleukin-4 (IL-4), IL-5, IL-6, IL-10, and IL-13 (Rabinovitch, 1994; Liblau et al., 1995).

Cell transfer experiments indicate that $CD4^+$ cells initiate IDDM and that Th1 cells may be effector cells of disease in NOD mice, although $CD8^+$ cells may also play an effector role and be responsible for the final destruction of islet β cells. The role of $CD8^+$ T cells in IDDM in NOD mice has been addressed in a number of ways. It has been shown that β2-microglobulin (β2m)–deficient NOD mice (NOD-$\beta2m^{null}$) lacking MHC class I molecules, and hence $CD8^+$ T cells, do not develop IDDM or insulitis (Serreze et al., 1994; Wicker et al., 1994). Expression of a β2m transgene in NOD-$\beta2m^{null}$ mice resulted in reconstitution of IFNγ-inducible cell-surface MHC class I protein on islet β cells. These mice developed insulitis but did not develop IDDM. These studies demonstrate that β2m expression and cell-surface MHC class I expression on islet β cells are essential for the initiation of IDDM in the NOD mouse and further confirm that efficient progression to diabetes requires both $CD4^+$ and $CD8^+$ T cells (Kay et al., 1996, 1997).

Furthermore, $CD8^+$ islet cell–specific cytolytic T cell lines and clones from NOD mice can transfer IDDM to irradiated NOD mice if coinjected with nondiabetogenic $CD4^+$ spleen T cells (Christianson et al., 1993; Wang et al., 1996). Therefore, $CD8^+$ T cells as final effector cells in IDDM require signals from $CD4^+$ T cells to effect β cell damage. However, in some cases when islet-reactive $CD8^+$ T cells are adoptively transferred to irradiated female NOD or NOD.SCID mice, IDDM occurs very rapidly and without $CD4^+$ T cells (Wong et al., 1996, 1997). TCR transgenic NOD mice with a $CD4^+$ T cell repertoire highly skewed for an anti-islet cell reactivity do not develop insulitis upon β2m deletion or treatment with anti-CD8 antibody (Wang et al., 1996). These results also suggest that $CD8^+$ cells are required for effective priming and expansion of autoreactive $CD4^+$ cells in TCR transgenic mice.

Evidence consistent with the presence of regulatory $CD4^+$ T cells in prediabetic NOD mice is provided by reports that cyclophosphamide induces acute IDDM (Charlton et al., 1989); sublethal irradiation is required to transfer disease by diabetogenic T cells (Wicker et al., 1986); adoptive transfer of IDDM can be blocked by cotransfer of $CD4^+$ T cells from young nondiabetic males (Hutchings and Cooke, 1990) or females (Boitard et al., 1989); and thymectomy and CD4 depletion potentiates the development of IDDM in NOD males (Sempé et al., 1994).

Curiously, several investigators have found that numerous T cell abnormalities emerge in NOD mice (Table 1) in an age-related manner. Most notably, these abnormalities are manifest by 4–6 weeks of age, coincident with the time of onset of periinsulitis, and include T cell proliferative hyporesponsiveness upon TCR stimulation (Zipris et al., 1991b), reduced IL-2 and IL-4 secretion in response to T cell activation (Rapoport et al., 1993a), and loss of regulatory T cell or suppressor T cell function (Bergerot et al., 1997). Another important change in T cell immunoregulation that occurs by the age of 4–6 weeks in NOD mice is a skewing toward Th1 cells, as reflected by the cytokine secretion profiles of islet-infiltrating T cells (reviewed by Bach et al., 1997; Bergerot et al., 1997). At this age, a high ratio of IFNγ/IL-4 expression can be found in the islet-infiltrated T cells of female NOD mice, and this ratio is predictive both of the onset of destructive insulitis and of a high incidence of IDDM in NOD females (Fox and Danska, 1997). In contrast, a high ratio of IL-4/IFNγ expression is detected in the islet-infiltrated T cells of male NOD mice, and the predominant IL-4 expression at the onset of islet inflammation predicts the onset of a nondestructive insulitis and a low incidence of IDDM in male NOD mice. Thus, it is plausible that these variations in cytokine secretion may elicit a Th1/Th2 imbalance in 4- to 6-week-old NOD mice. More significantly, this pattern of differential cytokine expression in young female NOD mice may explain why several types of treatment of IDDM, including autoantigen-induced tolerance (Elliot et al., 1994; Tian et al., 1996), cytokine (IL-4)–mediated therapy (Rapoport et al., 1993a; Mueller et al., 1996; Cameron et al., 1997a), and costimulation (CD28/B7)–mediated (Lenschow et al., 1996; Arreaza et al., 1997) therapy, are most effective when administered to NOD mice beginning at 2–3 weeks of age.

What causes these early changes in T cell immunoregulation in young NOD mice? It is possible that these changes are induced by a bacterial infection. Both IFNγ expression and Th1 cell development are stimulated by IL-12. The administration of IL-12 induces the rapid onset of IDDM, and the pancreatic expression of IL-12 correlates with IDDM development in NOD mice (Trembleau et al., 1995). Several bacterial products are enhanced by bacterial infection, including lipopolysaccharide and bacterial DNA, which potently induce IL-12 production by macrophages. Thus, an adverse bacterial infection in a young NOD mouse may stimulate the production of IL-12, upset the Th1/Th2 balance in favor of a dominant Th1 milieu, and thereby elicit the onset of insulitis and IDDM. Further experimentation will be needed to determine the relevance of this interesting scenario.

Another scenario, which may account for the age-related decline in regulatory $CD4^+$ T cell function in NOD mice, is an induced state of anergy in young NOD mice. If regulatory Th2 cells protect against IDDM, then the induction of anergy in regulatory Th2 cells may elicit disease. TCR ligation-induced anergy is manifested by T cells in both the thymus and periphery of NOD mice. This T cell anergy, which is first detectable at the onset of insulitis and persists until the development of IDDM in NOD mice, is mediated by a large reduction in IL-2 and the virtual absence of IL-4 secretion (Zipris et al., 1991b; Rapoport et al., 1993a). Complete reversal of this NOD T cell anergy and complete prevention of destructive insulitis and IDDM can be achieved by the systemic administration of either IL-4 (Rapoport et al., 1993a; Cameron et al., 1997a, 1997b) or an anti-CD28 monoclonal antibody (Arreaza et al., 1997) or by immunostimulation in vivo with adjuvants (Qin et al., 1993). Each of these treatments preferentially stimulates the intra-islet production of IL-4, a Th2-derived cytokine required for polarization of T cells to the Th2 subset. A significant proportion of regulatory $CD4^+$ Th2-like cells may therefore be anergic in NOD mice, a notion that is compatible with the recently proposed hypothesis that "regulatory

Th2 cell anergy" can mediate the pathogenesis of IDDM (Salojin et al., 1997a). Thus, as mentioned above, if young NOD mice become susceptible to bacterial infection and this infection enhances the production of IL-12, the increase in IL-12 concentration may down-regulate IL-4 activity and lead to the anergy of IL-4–secreting Th2 cells.

It appears that the function of Th2 cells may be compromised in young NOD mice to a greater extent than that of Th1 cells, possibly because Th2 cells may possess a higher activation threshold than Th1 cells. Hence, regulatory Th2 cells in NOD mice may be insufficiently stimulated mice to down-regulate diabetogenic Th1 cells reactive against islet β cell autoantigens. Such a possibility could explain the proposed Th1/Th2 paradigm in IDDM (Liblau et al., 1995; André et al., 1996; Nicholson and Kuchroo, 1996), in which functionally active Th2 cells protect against disease. Strong evidence for this conclusion is lacking, however, since data both in favor and against this paradigm have been obtained. While NOD islet infiltrate–derived Th1 cells reactive to either insulin, GAD65, or another unknown islet autoantigen (Daniel et al., 1994; Katz et al., 1995; Daniel and Wegmann, 1996) and spleen-derived insulin-reactive Th1 cells (Daniel and Wegmann, 1996) can transfer IDDM, islet-derived $CD4^+$ Th1 cells can also prevent IDDM (Chosich and Harrison, 1993; Akhtar et al., 1995; Tan et al., 1996). Similarly, while IL-4–secreting Th2 cells (Bergerot et al., 1994; Ploix et al., 1997) and/or Th3 cells (secreting IL-4, IL-10, and transforming growth factor-β) obtained from the intestinal mucosa of oral insulin–fed mice transfer protection from IDDM (Chen et al., 1994), islet-derived autoantigen specific Th2 cells do not transfer protection from IDDM (Katz et al., 1995). Rather, the latter Th2 cells directly transfer a general pancreatitis into immunocompromised NOD.SCID mice but not neonatal NOD mice (Pakala et al., 1997). In addition, two insulin-reactive and two GAD65-reactive splenic Th2 cell clones each elicited IDDM upon transfer into NOD.SCID but not neonatal NOD mice (Daniel and Wegmann, 1996). Further investigation will reveal whether the exceptions to the Th1 effector paradigm (promotes IDDM) and the Th2 regulatory paradigm (protects from IDDM) are attributable to differences in the tissue of origin, antigen specificity, ability to home to pancreatic islets, level and duration of cytokine production, ability to be regulated by interacting T cells and APCs, or other factors (e.g., bacterial infection) in the various Th1 and Th2 cell populations examined.

How Can Regulatory $CD4^+$ Th2 Cells Become Progressively Unresponsive and Ineffective in the Face of Autoreactive Th1 Cells?

If autoimmunity develops from a simple failure of negative selection in the thymus, it is difficult to explain why there is little evidence of autoimmune reactivity in the pancreas of the NOD mouse throughout the first 3–4 weeks of life (André et al., 1996). This outcome may arise from a deficit in regulatory $CD4^+$ Th2 cell function. This deficit might be regulated by early events in T cell differentiation, which may elicit a dominance in the number and function of diabetogenic Th1 cells relative to regulatory Th2 cells. NK-like thymocytes and peripheral T cells (NK-T cells), which proliferate in response to the CD1 MHC class I–like ligand, are believed to be a major T cell source of IL-4 for the development of Th2 cells (Bendelac et al., 1997). Interestingly, the number and function of NK-T cells is diminished about 3-fold in the thymus and periphery of NOD mice at 3 weeks of age, and anti-CD3–induced IL-4 secretion is barely detectable until 8 weeks of age (Gombert et al., 1996a). IL-7 plays a crucial role in the functional maturation of NK-T cells (Vicari et al., 1996) and restores IL-4 production by stimulated mature NK-T cells in the thymus and spleen (Gombert et al., 1996b). The NOD NK-T cell defect therefore may arise from insufficient IL-7 bioavailability, which contributes to reduced IL-4 production by activated NOD T cells (Rapoport et al., 1993a; Cameron et al., 1997a, 1997b). This deficiency in IL-4 production could ultimately generate an imbalance between Th1 and Th2 cells, in favor of Th1 cells, in the periphery. Indeed, administration of IL-7 to NOD mice protects them from IDDM, and this protective effect is mediated by the ability of IL-7 to restore the differentiation, function, and deficit of IL-4–producing T cells in NOD mice (Gombert et al., 1996b).

Thus, a relative lack of IL-4 production by NK-T cells is associated with and may be causal to the onset of IDDM. This idea is further supported by the decreased frequency of IL-4–producing NK-T cells and the impaired IL-4 production by circulating T cells in the peripheral blood of patients with IDDM (Berman et al., 1996; Wilson et al., 1997). It appears that IDDM is associated with an extreme Th1 phenotype for NK-T cells. These correlations between the development of autoimmune T cell reactivity and deficient $CD4^+$ NK-T cell activity and frequency in the NOD mouse and in patients with IDDM provide a strong framework for the hypothesis that IDDM results from a failure of immune regulation.

What is the Evidence That Immune Dysregulation Influences the Onset of IDDM?

Despite the attraction of this "failure of immune regulation" hypothesis, the subject of whether NK-T cells are involved in the control of Th2 cell differentiation has been debated. While NK-T cells are not obligatory for all Th2-dependent responses (e.g., parasite- and antigen-specific responses as well as IgE production) in CD1-deficient mice (Brown et al., 1996; Bendelac et al., 1997; Smiley et al., 1997), T cell IL-4 secretion is markedly diminished in CD1-deficient mice (Chen et al., 1997; Mendiratta et al., 1997; Smiley et al., 1997). NK-T cells are diminished in number and decreased in frequency prior to the onset of disease in several murine models of autoimmunity (Takeda and Dennert, 1993; Gombert et al., 1996b; Mieza et al., 1996; Vicari and Zlotnik, 1996; Bendelac et al., 1997). In these models, autoimmunity is temporally accelerated by depletion of NK-T cells. IDDM in NOD mice may also be prevented by adoptive transfer of a cell population containing NK-T cells (Baxter et al., 1997). These sets of data are consistent with the notions that differentiation of T cells into IL-4–secreting Th2 cells requires IL-4 priming and that IL-4 produced by NK-T cells stimulates Th2 cell differentiation and protects from autoimmune disease.

An alternative explanation has been proposed for the mechanism of action of NK-T cells. Rather than contributing solely to the generation of Th2 cells, NK-T cells may expand or maintain the survival or function of regulatory Th2 cells and down-regulate islet-infiltrating effector Th1 cells (Bach et al., 1997). A relative absence of NK-T cells during the first few weeks of life of an NOD mouse may upset this Th1/Th2 balance, initiate events that lead to immune dysregulation, and thereby influence the onset of IDDM.

Immune dysregulation and the onset of IDDM may result from several functional deficiencies in NOD mice (Table 1), which may generate an imbalance between autoimmune islet β cell–reactive T cells and the factors (cells and cytokines) that normally keep these T cells in check (André et al., 1996; Arreaza et al., 1996; Bergerot et al., 1997). At the onset of insulitis, the number of $CD4^+$ T cells in NOD peripheral lymphoid organs increases (Zhang et al., 1994), and this increase subsides after the onset of IDDM (Zipris et al., 1991a). Coincident with the appearance of insulitis at about 4–6 weeks of age, a defective syngeneic mixed lymphocyte reaction (SMLR) response is detectable in NOD mice (Bergerot et al., 1997). This age-related defect resides in an SMLR responder spleen and mesenteric lymph node–derived $CD4^+$ T cell population (Bergerot et al., 1997), is characterized by reduced IL-2 production by these T cells (Serreze and Leiter, 1988), and correlates closely with increased progression to IDDM (Baxter et al., 1989). These observations may explain why splenic $CD4^+$ T cells from only young (younger than 4–5 weeks old) NOD female mice are able to suppress or delay the transfer of IDDM (Boitard et al., 1989). In an SMLR response, T cells proliferate in response to self-MHC class II, inducing the activation of regulatory T cells. As a result of the weak peptide-binding properties of $I\text{-}A^g$ molecules, the affinity of T cells for $I\text{-}A^g$ molecules on interacting APCs in NOD mice may be too low to trigger the secretion of normal levels of several cytokines, including IL-2 and IL-4, and to generate functionally competent regulatory $CD4^+$ T cells. Thus, a deficiency in, rather than an absence of, regulatory $CD4^+$ T cells is manifested during an SMLR response in NOD mice greater than 6 weeks of age (Bergerot et al., 1997). A similar peripheral immunoregulatory defect occurs in patients with IDDM (Bowman et al., 1994).

The observation that NOD mice possess defective SMLR responses indicates that they possess a more global defect in $CD4^+$ T cell–mediated suppression, and not only a loss of T cell specific tolerance to Islet β cell autoantigens (Bergerot et al., 1997). Interestingly, all NOD mice, irrespective of age, sex, and disease progression, possess islet cell–reactive $CD4^+$ T cells in peripheral lymph nodes, and similar reactivity occurs in non–diabetes-prone mouse strains (Burtles et al., 1992). In these strains as well as in NOD mice greater than 6 weeks of age, cells from nonislet tissues fail to activate syngeneic T cells in an SMLR. The onset of deficient regulatory $CD4^+$ T cell function at an early age may disrupt the T cell balance required to maintain self-tolerance and thereby augment early T cell autoreactivity to islet cell autoantigens (Kaufman et al., 1993; Tisch et al., 1993). Such an imbalance may elicit a loss of immunoregulation of pathogenic islet-reactive T cells and trigger the development of destructive insulitis and IDDM according to a model shown in Figure 1. Thus, young nondiabetic NOD mice possess normal levels of functional regulatory $CD4^+$ T cells until about 4–6 weeks of age, and then this T cell function declines rapidly as the first islet antigen–autoreactive T cells are detected.

An important cytokine that may be involved in the regulation of some of the age-related functional deficiencies of NOD T cells is TNFα. TNFα increases T cell autoreactivity to Islet cells and exacerbates IDDM when administered in low doses from birth to 3 weeks during neonatal life in NOD mice, while administration of anti-TNFα during this same neonatal period completely prevents the development of IDDM (Yang et al., 1994). In contrast, the administration of TNFα to adult NOD mice (≥6 weeks of age) blocks the development of IDDM, whereas anti-TNFα exacerbates IDDM in adult NOD mice.

How may these paradoxical age-related differences in susceptibility and resistance to IDDM by TNFα treatment be explained? It is known that chronic TNFα exposure can down-regulate T cell effector function (decreased proliferation and reduced Th1 and Th2 cytokine production), while chronic anti-TNFα exposure, by blocking engenous TNFα, can up-regulate antigen-specific T cell responses and therefore up-regulate T cell effector function (Cope et al., 1997a). Based on these findings, it has been proposed that TNFα in neonatal mice may act as a growth factor for T cells in the thymus, specific for both self and foreign antigens; augment peripheral T cell effector function by increasing the expression of integrins and selectins; and enhance the homing of activated T cells to the pancreas (Cope et al., 1997b). Anti-TNFα blocks these effects and prevents primary follicle and germinal center formation in lymph nodes. This anti-TNFα treatment presumably decreases autoreactive B cell formation and associated B cell APC function and may also interfere with the development and migration of autoreactive T cells to the pancreas.

Since chronic exposure to TNFα and anti-TNFα reduces and augments signaling through the TCR, respectively (Cope et al., 1997a), an alternate scheme to explain the effects of TNFα and anti-TNFα in neonatal and adult NOD mice has been hypothesized. According to the latter hypothesis, TNFα, which is constitutively expressed in the neonatal thymus, may decrease TCR signaling and negative selection and increase the number of autoreactive T cells that migrate to the periphery and possibly also the pancreas in a neonatal NOD mouse (Cope et al., 1997b). These effects may be blocked neonatally by anti-TNFα and lead to increased negative selection and protection from IDDM. In an adult NOD mouse, TNFα-mediated reduction in TCR signaling may decrease autoreactive T cell effector function and inhibit the onset of IDDM, whereas anti-TNFα may exacerbate IDDM by increasing TCR signaling and stimulate autoreactive T cell effector function. Thus, in an age-dependent manner, endogenous TNFα might be able to alter the thresholds required for negative and positive T cell selection in the thymus and in this way shape the autoreactive T cell repertoire and susceptibility or resistance

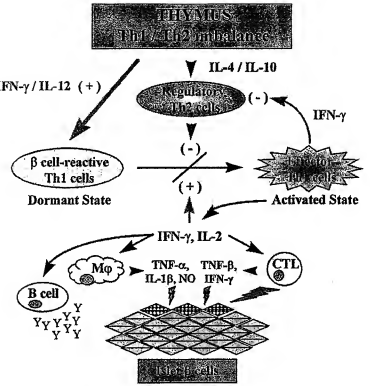

Figure 1. Model of Immune Dysregulation of T Cells Leading to Islet β Cell Destruction and Onset of IDDM in NOD Mice

Immunologic self-tolerance to pancreatic β cells is normally maintained by $CD4^+$ regulatory Th2 T cells, which suppress the activation of $CD4^+$ autoreactive Th1 T cells. In the NOD mouse, a Th1/Th2 imbalance occurs in the thymus and periphery and leads to a progressive, age-dependent elimination of function of regulatory Th2 T cells. Autoreactive Th1 T cells become activated and mediate pancreatic islet β cell destruction by participating in the recruitment of activated macrophages (Mφ) and cytotoxic T lymphocytes (CTLs), and these Th1 cells also help B cells to produce IgG2a autoantibodies (Y) against islet β cell autoantigens. Finally, the loss of Th2 T cell-mediated immunoregulation leads to a spreading of autoreactivity to islet β cell autoantigens that ultimately results in the onset of IDDM. (+), positive regulation; (−), negative regulation.

to IDDM. Studies of the effects of TNFα on negative and positive selection and of the possible role of this cytokine in controlling the function of regulatory $CD4^+$ Th2 cells as well as certain other cytokines, chemokines, and their specific receptors in the periphery are problems that merit further investigation.

Does the Lack of Costimulation by CD28 or CTLA-4 Mediate the Onset of IDDM?

Differential CD28-B7 costimulation for Th1 or Th2 development may be controlled by at least two candidate non-MHC IDDM susceptibility loci. CTLA-4 is a negative regulator of T cell activation and autoreactivity, and a mutation in the CTLA-4 gene may be associated with susceptibility to IDDM in humans (Nistic et al., 1996; Todd and Farrall, 1996; Donner et al., 1997). The *Idd-5* non–MHC-linked diabetogenic locus, which colocalizes with the CD28 and CTLA-4 genes on mouse chromosome 1, controls resistance to cyclophosphamide-induced apoptosis of NOD lymphocytes (Colucci et al., 1997). The expression of CTLA-4 and CD28 is defective in NOD mice, suggesting that one or both of these molecules may be involved in the control of apoptosis resistance, thymocyte selection, and IDDM susceptibility. Consistent with these notions, administration of soluble CTLA-4-Ig to young NOD mice prevents IDDM (Lenschow et al., 1995), and IDDM is enhanced in CTLA-4-Ig transgenic NOD mice (Lenschow et al., 1996).

Coexpression of B7-1 with TNFα in the pancreas increases the incidence of IDDM in transgenic NOD mice compared with the expression of either one of these transgenes alone (Guerder et al., 1994), in agreement with the result that the TNFα gene is tightly linked to an IDDM susceptibility locus (Nistic et al., 1996). It stands to reason, therefore, that if the binding of islet autoantigen peptides to $I\text{-}A^{g7}$ preferentially up-regulates B7–1 expression, this may limit B7–2 expression and Th2 cell development as well as activate other factors that potentiate the onset of IDDM. This idea is supported by the observations that IDDM is exacerbated in CD28-deficient NOD mice (Lenschow et al., 1996) and that IDDM is prevented by the administration of an activating anti-CD28 monoclonal antibody to young (2–4 week-old) but not older (≥5 week-old) NOD mice (Arreaza et al., 1997). Thus, the coupled effects of peptide binding to $I\text{-}A^{g7}$, deficiency in CD28 signaling and impairment in negative regulation by CTLA-4 of T cell activation may result in the insufficient costimulation, higher threshold of activation, resistance to apoptosis, and associated defect in proliferation and function of regulatory Th2 cells found in NOD mice.

Predictably, the net outcome of these coupled effects is similar to that which occurs in CTLA-4 deficient mice: namely, the dysregulation of costimulation, leading to the strong activation of T cells that mediate tissue destruction and autoimmune disease. In NOD mice, the outcome is the progression from a nondestructive periinsulitis, which persists until about 10–13 weeks of age, to a very invasive insulitis and then destructive insulitis that within the next 3 weeks initiates the onset of IDDM in about 80% of females (Bach et al., 1997; Gazda et al., 1997; Lafferty, 1997). The possibility that an increase

in CTLA-4 expression by Th2 cells mediates the appearance of invasive and destructive insulitis deserves further consideration.

Does an IDDM-Susceptibility Locus Control the Progression from Nondestructive Insulitis to Destructive Insulitis and the Onset of IDDM?

Deficient regulatory T cell–dependent control of autoreactive effector T cells can elicit various autoimmune diseases depending on the genetic makeup of the host (Sakaguchi et al., 1996). Accordingly, the failure in T cell regulation and escape from islet β cell tolerance in NOD mice is also regulated by several (at least 16) IDDM-susceptibility and IDDM-resistant loci (Todd et al., 1991; Serreze and Leiter, 1994; Ikegami et al., 1995; Wicker et al., 1995; Denny et al., 1997). Is it possible to identify which of these loci are involved in the regulation of progression from nondestructive insulitis to destructive insulitis and the onset of IDDM?

A partial answer to this question has been provided by studies of the genetic control of the above-mentioned TCR-dependent NOD T cell proliferative hyporesponsiveness. This hyporesponsiveness trait of NOD T cells was found to colocalize with the *Idd-4* non-MHC diabetogenic locus, which maps to the central region of mouse chromosome 11 and includes the CC β-chemokine gene family (Gill et al., 1995). This proliferative defect is intrinsic to T cells and results from the reduced ability of NOD T cells to activate TCR-coupled protein kinase C– and Ras-mediated second messenger signaling pathways (Rapoport et al., 1993b; Salojin et al., 1997b). This may explain why IL-2 secretion is greatly reduced in TCR-stimulated NOD T cells, in keeping with the possibility that IL-2 may be the candidate non-MHC diabetogenic gene in the *Idd-3* locus on mouse chromosome 3 (Denny et al., 1997). Preliminary analyses suggest that a relatively high level of intrapancreatic expression of the MIP-1β chemokine and low level of the MIP-1α chemokine is associated with nondestructive peri-insulitis, whereas high intrapancreatic concentrations of MIP-1β and low concentrations of MIP-1β are associated with the stages of invasive and destructive insulitis (Cameron et al., 1997b). These chemokine profiles appear to demarcate Th2- and Th1-mediated immune responses, respectively (Taub et al., 1996), and, based on the Th1/Th2 paradigm, may offer an explanation of how different relative levels of intrapancreatic expression of certain β chemokines can either prevent or exacerbate IDDM.

Conclusions and Future Perspectives

Considerable evidence obtained during the past 15 years suggests a major role for T cell immune dysregulation in the initiation of IDDM in NOD mice. T cell anergy and deficient T cell–mediated suppression may mediate susceptibility to IDDM in NOD mice, and Th2 cell anergy may be responsible for a failure in immune regulation. The key to the onset of immune dysregulation and aberrant increase in the number of autoreactive T cells in the periphery may be that the activation threshold required for TCR-stimulation is markedly increased in T cells from NOD mice and humans with IDDM. This increase in the number of peripheral T cells may arise from the weak peptide-binding affinity of I-A^{g7} molecules on NOD APCs and the resulting reduced capacity of APCs in the thymus of NOD mice to negatively select T cells with potential reactivity to islet autoantigens. While unresponsiveness to peptide/I-A^{g7} complexes may preclude stimulation of autoreactive T cells to a sufficiently high threshold level to induce their deletion, the levels of activation reached by these T cells may suffice to render them anergic to subsequent TCR stimulation. Nonetheless, this anergic state resulting from a failure of central and peripheral tolerance mechanisms remains capable of maintaining the autoimmune phenotype of these T cells. These T cells may still retain the capacity to initiate and contribute to the development of autoimmune disease.

Numerous fundamental questions related to IDDM remain to be explored in the NOD mouse model, and include the following. (1) Are I-A^{g7} MHC class II molecules underexpressed on the cell surface of APCs, and if so, is this the result of their generally low peptide-binding affinity and inherent instability? Does this weak binding affinity for peptides mediate the positive selection and exit into the periphery of an increased number of islet autoantigen reactive T cells? (2) What are the critical autoantigens in IDDM, and is there a primary autoantigen that induces the onset of IDDM? (3) What are the mechanisms of induction of islet β cell death, and which pathways (e.g., Fas/FasL, TNF/TNF receptor, or perforin) are most relevant to β cell apoptosis? (4) What agents, internal or external (e.g., viral, bacterial, or diet), trigger the onset of inflammation and IDDM? (5) What is the antigenic specificity and mechanism of action of regulatory T cells that may mediate protection from IDDM? (6) Finally, which cytokines and chemokines are most active in the down-regulation of the autoimmune response, and what is their mechanism of action? These and other questions will direct our exploration of the mechanisms underlying IDDM and other autoimmune diseases.

Selected Reading

Akhtar, I., Gold, J.P., Pan, L.Y., Ferrara, J.L.M., Yang, X.D., Kim, J.I. and Tan, K.N. (1995). J. Exp. Med. *182*, 87–97.

André, I., Gonzalez, A., Wang, B., Katz, J., Benoist, C., and Mathis, D. (1996). Proc. Natl. Acad. Sci. USA *93*, 2260–2263.

Arreaza, G.A., Cameron, M.J., and Delovitch, T.L. (1996). Clin. Immunother. *4*, 251–260.

Arreaza, G.A., Cameron, M.J., Jaramillo, A., Gill, B.M., Rapoport, M.J., Zucker, P., Chensue, S.W., Chakrabarti, S., Qin, H.-Y., Singh, B., et al. (1997). J. Clin. Invest. *100*, 2243–2253.

Ashton-Rickhardt, P., and Tonegawa, S. (1994). Immunol. Today *15*, 362–366.

Atkinson, M.A. (1997). Res. Immunol. *148*, 301–306.

Atkinson, M.A., Bowman, M.A., Campbell, L., Darrow, B.L., Kaufman, D.L., and Maclaren, N.K. (1994). J. Clin. Invest. *94*, 2125–2129.

Bach, J.F. (1994). Endocrine Rev. *15*, 516–542.

Bach, J.F., and Mathis, D. (1997). Res. Immunol. *148*, 285–286.

Bach, J.F., Chatenoud, L., Herbelin, A., Gombert, J.M., and Carnaud, C. (1997). Res. Immunol. *148*, 332–338.

Baxter, A.G., Adams, M.A., and Mandel, T.E. (1989). Diabetes *38*, 1296–1300.

Baxter, A.G., Kinder, S.J., Hammond, K.L., Scollay, R., and Godfrey, D.I. (1997). Diabetes *46*, 572–582.

Bendelac, A., Carnaud, C., Boitard, C., and Bach, J.F. (1987). J. Exp. Med. *166*, 823–832.

Bendelac, A., Rivera, M.N., Park, S.H., and Roark, J.H. (1997). Annu. Rev. Immunol. *15*, 535–562.

Bergerot, I., Fabien, N., Maguer, V., and Thivolet, C. (1994). J. Autoimmunity *7*, 655–663.

Bergerot, I., Arreaza, G.A., Cameron, M.J., Chou, H., and Delovitch, T.L. (1997). Res. Immunol. *148*, 348–358.

Berman, M.A., Sandborg, C.I., Wang, Z., Imfeld, K.L., Zaldivar, F., Dadufalza, V., and Buckingham, B.A. (1996). J. Immunol. *157*, 4691–4696.

Boitard, C., Yasunami, R., Dardenne, M., and Bach, J.F. (1989). J. Exp. Med. *169*, 1669–1680.

Bowman, M.A., Leiter, E.H., and Atkinson, M.A. (1994). Immunol. Today *15*, 115–120.

Brown, D.R., Fowell, D.J., Corry, D.B., Wynn, T.A., Moskowitz, N.H., Cheever, A.W., Locksley, R.M., and Reiner, S.L. (1996). J. Exp. Med. *184*, 1295–1304.

Buckner, J., Kwok, W.W., Nepom, B., and Nepom, G.T. (1996). J. Immunol. *157*, 4940–4945.

Burtles, S.S., Trembleau, S., Drexler, K., and Hurtenbach, U. (1992). J. Immunol. *149*, 2185–2193.

Cameron, M.J., Arreaza, G.A., Hardy, D., Zucker, P., Chensue, S.W., Chakrabarti, S., and Delovitch, T.L. (1997a). J. Immunol. *159*, 4686–4692.

Cameron, M.J., Arreaza, G.A. and Delovitch, T.L. (1997b). Crit. Rev. Immunol., in press.

Carrasco-Marin, E., Shimizu, J., Kanagawa, O., and Unanue, E.R. (1996). J. Immunol. *156*, 450–458.

Carrasco-Marin, E., Kanagawa, O., and Unanue, E.R. (1997). Res. Immunol. *148*, 291–301.

Charlton, B., Baceji, A., Slattery, R.M., and Mandel, T.E. (1989). Diabetes *38*, 441–447.

Chen, Y., Kuchroo, V.K., Inobe, J.I., Hafler, D.A., and Weiner, H.L. (1994). Science *265*, 1237–1240.

Chen, Y-H., Chiu, N.M., Mandal, M., Wang, N., and Wang, C.-R. (1997). Immunity *6*, 459–467.

Chosich, N., and Harrison, L.C. (1993). Diabetologia *36*, 716–721.

Christianson, S.W., Shultz, L.D., and Leiter, E.H. (1993). Diabetes *42*, 44–55.

Cohen, I.R. (1997). Res. Immunol. *148*, 286–291.

Colucci, F., Bergman, M.L., Goncalves, C.P., Cilio, C.M., and Holmberg, D. (1997). Proc. Natl. Acad. Sci. USA *94*, 9870–8674.

Conrad, B., Weidermann, E., Trucco, G., Rudert, W.A., Behboo, R., Ricordi, C., Rodriquez-Rilo, H., Ginegold, D., and Trucco, M. (1994). Nature *371*, 351–355.

Conrad, B., Weissmahr, N.R., Böni, J., Arcari, R., Schüpbach, J., and Mach, B. (1997). Cell *90*, 303–313.

Cope, A., Libiau, R.S., Yang, X.-D., Congia, M., Laudanna, L., Schreiber, R.D., Probert, L., Kollias, G., and McDevitt, H.O. (1997a). J. Exp. Med., in press.

Copa, A., Ettinger, R., and McDevitt, H.O. (1997b). Res. Immunol. *148*, 307–312.

Daniel, D., and Wegmann, D. (1996). Proc. Natl. Acad. Sci. USA *93*, 956–960.

Daniel, D., Gill, R.G., Schloot, N., and Wegmann, D. (1994). Eur. J. Immunol. *25*, 1056–1062.

Denny, P., Lord, C., Hill, N.J., Goy, J.V., Levy, E.R., Podolin, P., Peterson, L.B., Wicker, L.S., Todd, J.A., and Lyons, P.A. (1997). Diabetes *46*, 695–700.

Donner, H., Rau, H., Walfish, P.G., Braun, J., Siegmund, T., Finke, R., Herwig, J., Usadel, K.H., and Badenhoop, K. (1997). J. Clin. Endocrinol. Metab. *82*, 143–146.

Elias, D., Marcus, H., Reshef, T., Ablamunits, V., and Cohen, I.R. (1995). Eur. J. Immunol. *25*, 2851–2857.

Elias, D., Meilin, A., Ablamunits, V., Birk, O.S., Carmi, P., Konen-Waisman, S., and Cohen, I.R. (1997). Diabetes *46*, in press.

Elliot, J.F., Qin, H.Y., Bhatti, R., Singh, R., Smith, D.K., Lauzon, J., and Singh, B. (1994). Diabetes *43*, 1494–1499.

Forrest, J.M., Menser, M.A., and Burgess, J.A. (1971). Lancet *2*, 332–334.

Fox, C.J., and Danska, J.S. (1997). J. Immunol. *158*, 2414–2424.

Galley, K.A., and Danska, J.S. (1995). J. Immunol. *154*, 2969–2982.

Gamble D. R., Taylor, K.W., and Cumming, H. (1973). Br. J. Med. *4*, 260–262.

Gaskins, H.R., Prochazka, M., Hamaguchi, K., Serreze, D.V., and Leiter, E.H. (1992). J. Clin. Invest. *90*, 2220–2227.

Gazda, L.M., Charlton, B., and Lafferty, K.J. (1997). J. Autolmmun. *10*, in press.

Gill, B.M., Jaramillo, A., Ma, L., Laupland, K.B., and Delovitch, T.L. (1995). Diabetes *44*, 614–619.

Graves, P.M., Norris, J., Pallansch, M.A., Gerling, I.C., and Rewers, M. (1997). Diabetes *46*, 161–168.

Gombert, J.M., Herbelin, A., Tancrede-Bohin, E., Dy, M., Carnaud, C., and Bach, J.F. (1996a). Eur. J. Immunol. *26*, 2989–2998.

Gombert, J.M., Tancrede-Bohin, E., Hameg, A., Do Carmo Leite-de-Moraes, M., Vicari, A., Bach, J.F., and Herbelin, A. (1996b). Int. Immunol. *8*, 1751–1758.

Guerder, S., Picarella, D.E., Linstey, P., and Flavell, R.A. (1994). Proc. Natl. Acad. Sci. USA *91*, 5138–5142.

Harrison, L.C., Demsey-Collier, M., Kramer, D.R., and Takahashi, K. (1996). J. Exp. Med. *184*, 2167–2174.

Harrison, L.C., Honeyman, M.C., Trembleau, S., Gregori, S., Gallazzi, F., Augstein, P., Brusic, V., Hammer, J., and Adorini, L. (1997). J. Exp. Med. *185*, 1013–1021.

Haskins, K., and McDuffie, M. (1990). Science *249*, 1433–1436.

Haskins, K., Portas, M., Bradley, B., Wegmann, D., and Lafferty, K. (1988). Diabetes *37*, 1444–1448.

Haskins, K., Portas, M., Bergman, B., Lafferty, K., and Bradley, B. (1989). Proc. Natl. Acad. Sci. USA *86*, 8000–8004.

Hutchings, P.R., and Cooke A. (1990). J. Autolmmunity *3*, 175–185.

Ikegami, H., Makino, S., Yamato, E., Kawaguchi, Y., Ueda, H., Sakamoto, T., Takekawa, K., and Ogihara, T. (1995). J. Clin. Invest. *96*, 1936–1942.

Kaufman, D., Clare-Salzler, M., Tian, J., Forsthuber, T., Ting, G., Robinson, P., Atkinson, M.A., Sercarz, E., Tobin, A., and Lehmann, P. (1993). Nature *366*, 69–72.

Katz, J.D., Benoist, C., and Mathis, D. (1995). Science *268*, 1185–1188.

Kay, T.W., Parker, J.L., Stephens, L.A., Thomas, H.E. and Allison, J. (1996). J. Immunol. *157*, 3688–3693.

Kay, T.W., Chaplin, H.L., Parker, J.L., Stephens, L.A., and Thomas, H.E. (1997). Res. Immunol. *148*, 327–332.

Kikutani, H., and Makino. S. (1992). Adv. Immunol. *52*, 285–322.

Komagata Y., Masuko, K., Tashiro, F., Kato, T., Ikuta, K., Nishioka, K., Ito, K., Miyazaki, J., and Yamamoto, K. (1996). Int. Immunol. *8*, 807–814.

Lafferty, K. (1997). Res. Immunol. *148*, 313–319.

Lerger, E., Becourt, C., Bach, J.F., and Boitard, C. (1995). J. Exp. Med. *181*, 1635–1642.

Leiter, E.H., and Serreze, D.V. (1992). Reg. Immunol. *4*, 263–273.

Lenschow, D.J., Ho, S.C., Sattar, H., Rhee, L., Gray, G., Nabavi, N., Herold, K.C., and Bluestone, J.A. (1995). J. Exp. Med. *181*, 1145–1155.

Lenschow, D.J., Herold, K.C., Rhee, L., Patel, B., Koons, A., Qin, H.-Y., Fuchs, E., Singh, B., Thompson, C.B., and Bluestone, J.A. (1996). Immunity *5*, 285–293.

Libiau, R.S., Singer, S.M., and McDevitt H. O. (1995). Immunol. Today *16*, 34–38.

Lund, T., O'Reilly, L., Hutchings, P., Kanagawa, O., Simpson, E., Gravely, R., Chandler, P., Dyson, J., Picard, J.K., Edwards, A., et al. (1990). Nature *345*, 727–729.

Maeda, T., Sumida, T., Kurasawa, K., Tomioka, H., Itoh, I., Yoshida, S., and Koike, T. (1991). Diabetes *40*, 1580–1585.

Makino, S., Kunimoto, K., Maraoka, Y., Mizushima, Y., Katagiri, K., and Toshino, Y. (1980). Exp. Anim. *29*, 1–13.

Mendiratta, S.K., Martin, W.D., Hong, S., Boesteanu, A., Joyce, S., and Van Kaer, L. (1997). Immunity *6*, 469–477.

Mieza, M.A., Itoh, T., Cui, J.Q., Makino, Y., Kawano, T., Tsuchida, K., Kolke, T., Shirai, H., Yagita, H., Matsuwaza, A., et al. (1996). J. Immunol. *156*, 4035–4040.

Millich, D.R., Jones, J.E., McLachlan, A., Houghton, R., Thornton, G.B., and Hughes, J.L. (1989). J. Immunol. *143*, 3148–3156.

Mueller, R., Krahl, T., and Sarvetnick, N. (1996). J. Exp. Med. *184*, 1093–1099.

Muir, A., Peck, A.Clare-Salzler, M., Song, Y.-H., Cornelius, J., Luchetta, R., Krischer, J., and MacLaren, N. (1995). J. Clin. Invest. *95*, 628–634.

Nakano, N., Kikutani, H., Nishimoto, H., and Kishimoto, T. (1991). J. Exp. Med. *173*, 1091–1097.

Nepom, G.T., and Erlich, H. (1991). Annu. Rev. Immunol. *9*, 493–525.

Nicholson, L.B., and Kuchroo, V.K. (1996). Curr. Opin. Immunol. *8*, 837–842.

Nistic, L., Buzzetti, R., Pritchard, L.E., Van der Auwera , B., Giovanni, C., Bosi, E., Larrad, M.T.M., Rios, M.S., Chow, C.C., Cockram, C.S. et al. (1996). Hum. Mol. Genet. *7*, 1075–1080.

Noorchashm, H., Noorchashm, N., Kern J., Rostami, S.Y., Barker, C.F., and Naji, A. (1997). Diabetes *46*, 941–946.

Notkins, A.L., Onodera, T., and Prabhakar, B. (1984). In: Concepts in Viral Pathogenesis, A.L. Notkins and M.B.A. Oldstone, eds. (Heidelberg: Springer-Verlag), pp. 210–215.

Oldstone, M.B.A. (1988). Science *239*, 500–502.

Oldstone, M.B.A. (1997). Scand. J. Immunol., in press.

Pakala, S.V., Kurrer, M.O., and Katz, J.D. (1997). J. Exp. Med. *186*, 299–306.

Pilstrom, B., Bjork, L., and Bohme, J. (1995). Cytokine *7*, 806–814.

Ploix, C., Bergerot, I., Fabien, N., Perche, S., Moulin, V., and Thivolet, C. (1997). Diabetes *46*, in press.

Qin, H., Sadelain, M.W., Lauzon, J., and Singh, B. (1993). J. Immunol. *150*, 2072–2080.

Rabinovitch, A. (1994). Diabetes *43*, 613–621.

Rapoport, M.J., Jaramillo, A., Zipris, D., Lazarus, A.H., Serreze, D.V., Leiter, E.H., Cyopick, P., Danska, J.S., and Delovitch, T.L. (1993a). J. Exp. Med. *178*, 87–99.

Rapoport, M.J., Lazarus, A.H., Jaramillo, A., Speck, E., and Delovitch, T. L. (1993b). J. Exp. Med. *177*, 1221–1226.

Reich, E.P., von Grafenstein, H., Barlow, A., Swenson, K.E., Williams, K., and Janeway, C.A. (1994). J. Immunol. *152*, 2279–2288.

Reizis, B., Eisenstein, M., Bockova, J., Konen-Waisman, S., Mor, F., Elias, D., and Cohen, I.R. (1997). Int. Immunol. *9*, 43–51.

Ridgeway, W.M., Fasso, M., Lanctot, A., Garvey, C., and Fathman, C.G. (1996). J. Exp. Med. *183*, 1657–1662.

Roep, B.O. (1996). Diabetes *45*, 1147–1156.

Rohane, P.W., Shimada, A., Kim, D.T., Edwards C. T., Charlton B., Shultz L.D., and C. G. Fathman. (1995). Diabetes *44*, 550–554.

Sakaguchi, S., Toda, M., Asano, M., Itoh, M., Morse, S.S., and Sakaguchi, N. (1996). J. Autoimmunity *9*, 211–220.

Salojin, K., Zhang, J., Madrenas, J., and Delovitch. (1997a). Immunol. Today *19*, in press.

Salojin, K., Zhang, J., Cameron, M., Gill, B., Arreaza, G., Ochi, A., and Delovitch, T.L. (1997b). J. Exp. Med., *186*, 887–897.

Santamaria, P., Utsugi, T., Park, B-J., Averhill, N., Kawazu, S., and Yoon, J-W. (1995). J. Immunol. *154*, 2494–2503.

Schmidt, D., Verdaguer, J., Averill, N., and Santamaria, P. (1997). J. Exp. Med. *186*, 1059–1075.

Sempé, P., Richard, M.F., Bach, J.F., and Boitard, C. (1994). Diabetologia *37*, 337–343.

Sercarz, E.E., Lehmann, P.V., Ametani, A., Benichou, G., Miller, A., and Moudgil, K. (1993). Annu. Rev. Immunol. *11*, 729–766.

Serreze, D.V. (1993). FASEB J. *7*, 1092–1096.

Serreze, D.V., and Leiter, E.H. (1988). J. Immunol. *140*, 3801–3807.

Serreze, D.V., and Leiter, E.H. (1994). Curr. Opin. Immunol. *6*, 900–906.

Serreze, D.V., Gaskins, H.R., and Leiter, E.H. (1993a). J. Immunol. *150*, 2534–2543.

Serreze, D.V., Gaedeke, J.W., and Leiter, E.H. (1993b). Proc. Natl. Acad. Sci. USA *90*, 9625–9629.

Serreze, D.V., Leiter, E.H., Christianson, G.J., Greiner, D., and Roopenian, D.C. (1994). Diabetes *43*, 505–509.

Serreze, D.V., Chapman, H.D., Varnum, D.S., Hanson, M.S., Reifsnyder, P.C., Richard, S.D., Fleming, S.A., Leiter, E.H., and Schultz, L.D. (1996). J. Exp. Med. *184*, 2049–2053.

Shimada, A., Rohane, P., Fathman, C.G., and Charlton, B. (1996). Diabetes *45*, 71–78.

Simone, E., Danieri, D., Schloot, N., Gottleib, P., Babu, S., Kawasaki, E., Wegmann, D., and Eisenbarth, G.S. (1997). Proc. Natl. Acad. Sci. USA *94*, 2518–2521.

Singh, B., Prange, S., and Jevnikar, A.M. (1998). Semin. Immunol., in press.

Smiley, S.T., Kaplan, M.H., and Grusby, M.J. (1997). Science *275*, 977–979.

Sprent, J., Lo, D., Gao, K., and Ron, Y.Y. (1988). Immunol. Rev. *101*, 173–190.

Takeda, K., and Dennert, G. (1993). J. Exp. Med. *177*, 155–164.

Tan, K.N., Min, H.K., Pan, L., Ferrara, L.M., and Myrick, K.V. (1996). Int. Immunol. *8*, 689–699.

Taub, D.D., Longo, D.L., Turcovski-Corrales, S.M., Murphy, W.J., Key M.L. (1996). J. Immunol. *156*, 2095–2103.

Tian, J., Lehmann, P.V., and Kaufman, D.L. (1994). J. Exp. Med. *180*, 1979–1984.

Tian, J., Atkinson, M.A., Clare-Salzler, M., Herschenfeld, A., Forsthuber, T., Lehmann, P.V., and Kaufman, D. (1996). J. Exp. Med. *183*, 1561–1567.

Tisch, R., and McDevitt, H.O. (1996). Cell *85*, 291–297.

Tisch, R., Yang, X-D., Singer, S.M., Liblau, R.S., Fugger, L., and McDevitt, H.O. (1993). Nature *366*, 72–75.

Tivol, E.A., Schweitzer, A.N., and Sharpe, A. (1996). Curr. Opin. Immunol. *8*, 822–830.

Todd, J.A., and Farrall, M. (1996). Hum. Mol. Genet. *5*, 1443–1448.

Todd, J.A., Aitman, T.J., Cornall, R.J., Ghosh, S., Hall, J., Hearne, C. M., Knight, A.M., Love, J.M., McAleer, M.A., Prins, J.B., et al. (1991). Nature *351*, 542–547.

Toyoda, H., Redford, A., Magalong, D., Chan, E., Hosszufalusi, N., Formby, B., Teruya, M., and Charles, M.A. (1992). Immuol. Lett. *32*, 241–245.

Trembleau, S., Penna, G., Bosi, E., Mortara, A., Gately, M.K., and Adorini, L. (1995). J. Exp. Med. *181*, 817–821.

Vicari, A.P., and Zlotnik, A. (1996). Immunol. Today *17*, 71–76.

Vicari, A., Herbelin, A., Do Carmo Leite-de-Moraes, M., Von Freeden-Jeffry, U., Murray, R., and Zlotnik, A. (1996). Int. Immunol. *8*, 1759–1766.

von Herrath, M.G., and Oldstone, M. (1996). Curr. Opin. Immunol. *8*, 878–885.

von Herrath, M.G., and Oldstone, M. (1997). J. Exp. Med. *185*, 531–540.

Vyse, T.J., and Todd, J.A. (1996). Cell *85*, 311–318.

Wang, B., Gonzales, A., Benoist, C., and Mathis, D. (1996). Eur. J. Immunol. *26*, 1762–1769.

Waters, S.H., O'Neil, J.J., Melican, D.T., and Appel, M.C. (1992). Diabetes *41*, 308–312.

Weckerle, H.M., Bradl, C., Linington, C., Kaab, G., and Kojiova, K. (1996). Immunol. Rev. *149*, 231–243.

Wicker, L.S. (1997). J. Exp. Med. *186*, 973–975.

Wicker, L.S., Miller, B.J., and Mullen Y. (1986). Diabetes *35*, 855–860.

Wicker, L.S, Leiter, E.H, Todd, J.A., Renjilian, R.J., Peterson, E.,

Fischer, P.A., Podolin, P.L., Zijlstra, M., Jaenisch, R., and Peterson, L.B. (1994). Diabetes *43*, 500–504.

Wicker, L.S., Todd, J.A., and Peterson, L.B. (1995). Annu. Rev. Immunol. *13*, 179–200.

Wilson, S.B., Kent, S.C., Patton, K.T., Orban, T., Jackson, R.A., Exley, M., Porcelli, S., Schatz, D.A., Atkinson, M.A., Balk, S.P., et al. (1997). Nature, in press.

Wong, F.S., and Janeway, C.A., Jr. (1997). Res. Immunol. *28*, 327–332.

Wong, F.S., Visintin, I., Wen, L., Flavell, R.A., and Janeway, C.A. (1996). J. Exp. Med. *183*, 67–76.

Yang, X.-D., Tisch, R., Singer, S., Cao, Z.A., Liblau, R.S., Schreiber, R.S., and McDevitt, H.O. (1994). J. Exp. Med. *180*, 995–1004.

Yang, Y., Charlton, B., Shimada, A., Canto, R.D., and Fathman, C.G. (1996). Cell *4*, 189–194.

Zechel, M.A., Chaturvedi, P., and Singh, B. (1997). Res. Immunol. *148*, 338–348.

Zhang, J.A., Davidson, L., Eisenbarth, G., and Weiner, H.L. (1991). Proc. Natl. Acad. Sci. USA *88*, 10252–10256.

Zhang, Z.L., Constantinou, D., Mandel, T.E., and Georgiou, H.M. (1994). Autoimmunity *17*, 41–48.

Zipris, D., Crow, A.R., and Delovitch, T.L. (1991a). Diabetes *40*, 429–435.

Zipris, D., Lazarus, A.H., Crow, A.R., Hadzija, M., and Delovitch, T.L. (1991b). J. Immunol. *146*, 3763–3771.

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Insulin-Dependent Diabetes Mellitus

Review

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Introduction

Insulin-dependent diabetes mellitus (IDDM) is a multifactorial autoimmune disease for which susceptibility is determined by environmental and genetic factors. Inheritance is polygenic, with the genotype of the major histocompatibility complex (MHC) being the strongest genetic determinant. However, even in monozygotic twins, the concordance rate is only 50% (Barnett et al., 1981), indicating the importance of a number of as yet unidentified environmental factors (Castano and Eisenbarth, 1990). There is a north–south gradient in incidence of the disease with the highest incidence (1%–1.5% in Finland) being in northern Europe, with decreasing incidence in more southerly and tropical locations. Although this suggests the effect of infectious agents, in the nonobese diabetic (NOD) mouse, germ-free NOD mice have the highest incidence (nearly 100%) that has been seen in any NOD colony.

While MHC class II genotype is one of the strongest factors determining susceptibility to IDDM, it has long been apparent that susceptibility at MHC class II is a necessary but not sufficient predisposing genetic factor. Microsatellite analyses of genome-wide polymorphisms in multiplex IDDM families and in NOD crosses with nonsusceptible strains have identified many other genetic regions that also influence susceptibility. Thus, in the NOD mouse there are at least 15 other regions on 11 other chromosomes that contribute to genetic predisposition (Vyse and Todd, 1996 [this issue of *Cell*]). In man, linkage studies have suggested an even larger number (as many as 19) genetic regions determining IDDM susceptibility. For the most part, the genes determining susceptibility in each of these chromosomal regions have yet to be identified. Several of these regions also influence susceptibility to a murine counterpart of systemic lupus erythematosus and to a murine model of multiple sclerosis (Vyse and Todd, 1996).

IDDM in animal models is T cell mediated and requires the participation of both $CD8^+$, class I MHC restricted and $CD4^+$, class II MHC restricted T cells (Wicker et al., 1995). Extensive studies in rodent models have failed to identify the origins of the autoreactivity in IDDM, but demonstrate the importance of a number (8–10) of islet β cell–expressed proteins that are the targets of the autoimmune process in this disease (Table 1). Other studies have shown the important roles of several regulatory and proinflammatory cytokines, including interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin-4 (IL-4), and IL-10, as well as the importance of a number of accessory molecules (B7.1, B7.2) (Lenschow et al., 1995) and adhesion molecules (very late antigen 4) (Yang et al., 1993).

Studies of rodent models and preliminary studies in man have shown that the completion of β cell destruction can be considerably delayed or prevented by parenteral administration of β cell autoantigens—including insulin, glutamic acid decarboxylase (GAD), and heat shock protein 60 (HSP60). A number of studies have also shown that manipulation of cytokine networks by administration of specific cytokines or their antagonists can delay or prevent diabetes.

Together, these advances have set the stage for developing a complete molecular understanding of the pathogenesis of this autoimmune disease and for the design of rational and effective means of prevention. Prevention could then replace insulin therapy, which is effective but associated with long term renal, vascular, and retinal complications.

The Role of the Major Histocompatibility Complex

Extensive sequencing of MHC class II alleles in man, the NOD mouse, and the Bio-breeding rat, as well as the use of NOD mice transgenic for several MHC class II molecules, has revealed a complex interplay between alleles of the two major isotypes of MHC class II molecules (HLA [human leukocyte antigen] DR and DQ in man, and I-A and I-E in the mouse]) (Wicker et al., 1995). Susceptibility to type I diabetes is most strongly determined by DQ and I-Aβ chain alleles that encode serine, alanine, or valine at position 57 on both chromosomes (Acha-Orbea and McDevitt, 1987; Todd et al., 1987). DQβ and I-Aβ position 57 aspartic acid positive alleles mediate resistance to IDDM, which varies in degree with the sequence of other residues in the DQα and β chains. Expression of I-E (β chain position 57 aspartic acid positive) in the NOD mouse, and of DR B1 chains expressing aspartic acid at position 57, also mediates varying degrees of resistance to type I IDDM. Thus, HLA DR B1 alleles lacking aspartic acid at position 57 in Japanese patients are associated with a higher degree of susceptibility than Asp-57(*) HLA-DR B1 alleles (Ikegami et al., 1989, Diabetes, abstract). There is also evidence that MHC class I genotype may have a similar modifying effect (Ikegami et al., 1993).

These associations have now been extensively tested in many studies (Nepom and Erlich, 1991) and several exceptions have been noted. (Ikegami et al., 1989, Diabetes, abstract; Erlich et al., 1993). Results from these studies indicate that polymorphisms in the DQα chain, elsewhere in the DQβ chain, and in the DR B1 chain play an important modifying role. In some populations this can be shown to be due to similar sequence polymorphisms at DR B1 position 57, with aspartic acid negative alleles mediating susceptibility while aspartic acid positive alleles mediate resistance. (Ikegami et al., 1989, Diabetes, abstract; Cucca et al., 1995). There is

Table 1. Targets of the Autoimmune Response in IDDM

Autoantigen	Antibody	T Cell Responses[a]
Insulin	+	+
GAD65/67	+	+
ICA 105 (IA-2)	+	?
Carboxypeptides H	+	+
Peripherin	+	+
HSP60	+	+
p69	+	?
ICA 512	+	?
52 kDa Ag	+	?
Gangliosides	+	?
38 kDa secretory granule antigen	?	+

[a] For specific references, see Atkinson and Maclaren, 1993.

also evidence that a polymorphism at HLA-DR B1 position 74 can have a strong modifying affect on susceptibility (Cucca et al., 1995) (Table 2, this study).

Peptide elution studies by Ramensee et al. (1995) and Reich et al. (1994) have provided indirect support for the concept that HLA-DQ-, -DR, and I-A polymorphisms affect susceptibility to IDDM by selectively affecting the nature of the peptides presented to T cells by these class II molecules. These authors showed that peptides eluted from HLA-DR alleles that have or lack aspartic acid at HLA-DRβ57 bind overlapping but distinct sets of peptides. Thus HLA DR B1-04 alleles lacking aspartic acid at position 57 bind peptides with glutamic acid or aspartic acid at position P9 in the peptide (Table 3). This is presumably because the absence of aspartic acid at DR β1 position 57 leaves a conserved arginine at DRα79 free to interact with a negative charge at the carboxyl terminus of the peptide (Stern et al., 1994; Wucherpfennig and Strominger, 1995). In position 57 aspartic acid positive alleles, Asp-57 forms a salt bridge with α-Arg-79, and peptides with a negative charge at or near the carboxyl terminus of the peptide are not bound to any appreciable degree. (These data are derived from amino acid sequence studies of complex mixtures of peptides eluted from the respective alleles. It is likely that both types of allele, which are nearly identical in sequence elsewhere in the DR β1 chain, will also bind many of the same peptides).

Table 2. MHC Class II Sequence Polymorphisms in IDDM

Locus	Allele	Susceptible	Resistant
DQ B1	0201	Ala-57	
DQ B1	0302	Ala-57	
DQ B1	0303[a]		Asp-57
DQ B1	0301[a]		Asp-57
DQ B1	0502	Ser-57	
DQ B1	0602[c]		Asp-57
DR B1	0405	Ser-57	
DR B1	0403		Asp-57, Glu-74
DR B1	0401[b]	Asp-57	
I-Eβ	g7[c]		Asp-57
I-A	g7	Ser-57	
I-A	b[c]		Asp-57

[a] Neutral or weakly negative with respect to IDDM.
[b] Less susceptible than DRB1 0405.
[c] Strongly resistant to IDDM.

Table 3. Peptides Bound by DR4 Subtypes

DR4 Allele	Amino Acid(s) at Peptide Position 9
0401 β-Asp-57	Ala, Ser, Gln
0405 β-Ser-57	Asp, Glu

Considerable evidence (see below) indicates that islet β cell damage and destruction is mediated by islet antigen specific T helper type 1 (Th1) lymphocytes. The results cited above suggest that, while susceptible and resistant alleles can present many of the same peptides, susceptible alleles also present a distinct subset of peptides with a negative charge at position P9. These peptides, when bound by susceptible DQ and I-A alleles, may preferentially induce a Th1 response. In contrast, resistant alleles would be expected to present peptides that would elicit a predominant Th2 response. NOD mice expressing transgenic I-A alleles ($I\text{-}A^d$, $I\text{-}A^k$, $I\text{-}Ag^7$) with a mutation to aspartic acid at position 57 in A_β have a decreased or zero incidence of IDDM (see references in Quartey-Papafio et al., 1995). Cell transfer studies suggest this decreased incidence is the result of a predominant Th2 response to islet cell antigens (Singer, et al., 1993), but definitive proof for this interpretation is yet to be published. Competition between susceptible and resistant alleles for binding a critical diabetogenic peptide has been postulated as an alternative explanation for these data (Nepom, 1990; Quartey-Papafio et al., 1995). Support for the former hypothesis is seen in studies of IDDM families (Thai and Eisenbarth, 1993). Although DQB1 0602 (an IDDM-resistant allele) positive siblings of diabetics rarely develop diabetes, they can produce high titers of autoantibodies to several islet cell antigens. This indicates that resistant alleles do not cause resistance by inducing more complete self-tolerance to islet cell antigens than do the susceptible DQβ1 alleles. (Nepom, 1990; Erlich et al., 1993).

The results cited above bring us tantalizingly close to understanding how susceptible and resistant alleles mediate their effects. The issues raised can only be resolved when peptide epitopes derived from critical islet cell autoantigens have been identified and characterized with respect to their ability to elicit insulitis and IDDM-inducing T cells. The long list of antigens that are the target of an autoimmune response in both mouse and man (see below) means that the peptide epitopes derived from a number of islet cell autoantigens will have to be identified and characterized to achieve this goal.

The Autoantigens Targeted in IDDM

The strong association that exists between specific MHC class II alleles and disease susceptibility implies that the diabetogenic response is antigen driven. This is supported by the observation that T cells obtained from NOD mice in which the β cells have been ablated at an early age no longer have the capacity to adoptively transfer disease (Larger et al., 1995). Studies in the NOD mouse from the neonatal period until disease onset suggest that the diabetogenic response can be viewed as a series of stages culminating in massive β cell destruction and the establishment of overt diabetes. Peri-insulitis, first seen at 4–6 weeks of age, is characterized by

an accumulation of macrophages, dendritic cells, and B and T lymphocytes that enter the periductal areas but remain outside of the islet proper. At later time points, intra-insulitis develops and is characterized by the direct invasion of the islets by infiltrating cells, and is dependent on the recognition of β cell antigen(s) (Wicker et al., 1992). A temporal analysis of β cell reactivity in NOD mice suggests that only a few autoantigens are targeted in the early stages (Kaufman et al., 1993; Tisch et al., 1993). As intra-insulitis progresses, additional β cell destruction occurs, apparently resulting in the sensitization and recruitment of other β cell-specific T cells found in the periphery. Intra-insulitis per se, however, does not appear to be sufficient to drive the response to an overt diabetic state. This is suggested by studies in NOD mice transgenic for a pathogenic T cell receptor (TCR) that exhibit a highly aggressive form of intra-insulitis beginning abruptly at 3–4 weeks of age, yet the time of onset (18–20 weeks) and the frequency of overt diabetes in these animals is only marginally enhanced (Katz et al., 1993a). These 3 week and 18–20 week checkpoints may reflect the requirement for additional events in order to initiate insulitis and then to progress to overt diabetes. These events may depend on the outcome of interactions occurring between effector and regulatory T cells (see below) or sequential targeting of specific β cell autoantigens, or both.

Only in the past 5–7 years has the identity of most of the β cell autoantigens been determined. Despite this progress, little is known about the role these autoantigens may play in the disease process, i.e. whether they are in fact pathogenic. At present, conclusions regarding the possible role/importance of a given β cell autoantigen in IDDM are based upon two sources: first, observed correlations between autoantibody reactivity (and more recently T cell reactivity) and disease progression in man and in NOD mice, and second, studies determining whether the diabetogenic response in NOD mice can be modulated following treatment with the autoantigen or transfer of specific T cell clones, or both.

Using the above criteria, glutamic acid decarboxylase (GAD) is one of only three critical β cell autoantigens. GAD is an enzmye with two isoforms, GAD65 and GAD67, that catalyze the biosynthesis of the neurotransmitter γ-aminobutyric acid. The presence of anti-GAD antibodies in the sera of prediabetic individuals has proven to be a reliable predictive marker for progression to overt diabetes (Baekkeskov et al., 1990; Hagopian et al., 1993). T cell reactivity in IDDM patients can be detected to a region of GAD that contains homology to the Coxsackie B P2-C viral protein (Atkinson et al., 1994). The fact that Coxsackie B viral infections have been implicated in cases of IDDM has led to the intriguing hypothesis that recognition of GAD may be stimulated in some instances by a response to the virus.

NOD mice also exhibit antibody reactivity to GAD (and to insulin). Responses to GAD and insulin (but not to other β cell autoantigens such as HSP60, peripherin, and carboxypeptidase H) can be detected in animals at an age when minimal histological signs of islet inflammation are observed (Kaufman et al., 1993; Tisch et al., 1993). Anti-GAD reactivity is seen in some NOD mice that exhibit extensive intra-insulitis, yet remain diabetes free (Tisch et al., 1993). These observations suggest that recognition of GAD (and insulin, see below) occurs early in the disease process, and that anti-GAD reactivity may mediate initial events associated with intra-insulitis. NOD mice remain protected from diabetes when treated with GAD either at an age preceding islet inflammation or when exhibiting extensive intra-insulitis, providing functional evidence that GAD may have a critical role in the disease process (Kaufman et al., 1993; Tisch et al., 1993; Elliott et al., 1994). In these studies protection, at least in part, appears to be mediated through the induction of GAD-specific regulatory T cells that secrete lymphokines that nonspecifically suppress the diabetogenic response. To determine the relative contribution and precise role of anti-GAD reactivity in the disease process, experiments need to be done in which GAD-specific T cells are selectively tolerized by clonal deletion/anergy induction, to detect the effect this has on development of insulitis and IDDM.

Insulin is another β cell autoantigen that appears to have a critical role in the diabetogenic response. Anti-insulin autoantibodies can be detected in ~50% of recent-onset IDDM subjects and are most frequent in younger children who exhibit an enhanced rate of β cell destruction (Castano and Eisenbarth, 1990). Insulin is a key T cell target in that insulin B chain-specific $CD4^+$ T cell clones can accelerate diabetes in young NOD mice or adoptively transfer disease in NOD-*scid* mice (Daniel et al., 1995). Furthermore, oral or parenteral treatment of young NOD mice with whole insulin or insulin B chain, respectively, can protect animals from diabetes (Zhang et al., 1991; Muir et al., 1995). This protection again appears to be partially mediated through the induction of immunoregulatory T cells, so that the relative contribution of anti-insulin reactivity to the disease process is still not clear. In contrast to young NOD mice treated with GAD, animals receiving insulin or insulin-B chain continue to exhibit intra-insulitis, suggesting that anti-insulin reactivity may be necessary for more distal events in disease progression.

Additional autoreactivity seen during the development of human diabetes includes antibodies to two tryptic fragments with molecular masses of 37 and 40 kDa, derived from a β cell antigen. Autoantibodies against these fragments have been detected in 60% of newly diagnosed individuals and appear to identify a subgroup of IDDM patients who rapidly progress to diabetes (Christie et al., 1994). The recent discovery that the two tryptic fragments are derived from the putative tyrosine phosphatase IA-2 should aid in assessing T cell reactivity to the autoantigen and its possible role in the diabetogenic response (Passini et al., 1995). A protein designated as p69 has been shown to be an additional target of autoantibodies found in IDDM patients (Pietropaolo et al., 1993).

Autoantibodies and T cell reactivity specific for HSP60 have also been detected in NOD mice. Whether HSP60 is targeted in the human diabetogenic response remains unclear. However, treatment of NOD mice with HSP60 protects animals from disease (Elias et al., 1991). Moreover, it has been reported that treatment of hyperglycemic NOD mice with an HSP60-specific peptide can reestablish euglycemic blood levels (Elias and Cohen, 1994). Finally, HSP60-specific $CD4^+$ T cell lines have been shown to accelerate or block disease in NOD recipients (Elias et al., 1991).

Undefined components of the β cell secretory granule have been shown to be targeted by pathogenic CD4+ T cell clones established from NOD mice (Haskins and McDuffie, 1990) and by CD4+ T cell clones from IDDM patients (Roep et al., 1990).

Thus, a number of β cell autoantigens are recognized during the diabetogenic process. The task at hand is to distinguish those antigens that play a primary role in initiating the autoimmune process from those autoantigens that elicit an autoimmune response as a secondary event due to local inflammation. This might be achieved in animal studies in which the T cells specific for a given autoantigen are selectively tolerized, and the effect this has on insulitis and IDDM then determined. A sequential study over time of T cell reactivity in HLA identical siblings of diabetics, and in recent onset IDDM patients, may also provide further insight into the relative importance of a given autoantigen.

The T Cell Response in IDDM

Studies primarily in the NOD mouse have attempted to determine whether the repertoire of infiltrating T cells exhibit Vα or Vβ restriction. To date, there has been no consistent evidence indicating that restriction in Vα or Vβ usage exists among T cells found in the pancreas. However, a recent study has reported that, in two diabetic patients, preferential usage of the Vβ7 gene was detected in the infiltrating T cells (Conrad et al., 1994). This restriction was argued to be the result of T cell activation by an unidentified infectious agent encoding a superantigen within the islets.

Studies with NOD mice deficient in MHC class I or class II expression—and in turn devoid of CD8+ or CD4+ T cells, respectively—have demonstrated that both T cell subsets are required for islet infiltration and subsequent β cell destruction (Katz et al., 1993b; Serreze et al., 1994; Wicker et al., 1994). However, the respective contribution of each subset is presently not clear. Numerous studies have shown that CD4+ T cells alone are far more efficient in the adoptive transfer of disease than CD8+ T cells. The effectiveness of CD4+ T cells in transferring disease is most likely due to the secretion of lymphokines such as IFNγ and TNFα that are directly toxic to β cells and that recruit nonspecific effector cells to amplify the response. CD8+ T cells on the other hand, may have a more restricted role in the disease process. It has been suggested that CD8+ T cells are required to initiate β cell injury, which in turn could lead to the priming of CD4+ T cells and subsequent amplification of the response (Wicker et al., 1994). The lack of insulitis in class I–deficient NOD mice and the appearance of CD8+ T cells in the islets prior to CD4+ T cells (Jarpe et al., 1991) support this notion.

CD4+ T cell dominance in the diabetic process may reflect the critical role this subset has in regulating the immune system. CD4+ T cells can be divided into distinct subsets based on their cytokine profiles. These subsets of Th cells oppose one another through reciprocal down-regulatory effects mediated by their respective cytokines. Th1 cells, which secrete IL-2, IFNγ, and TNFα and predominantly support cell-mediated immunity, are believed to be the primary CD4+ T cells mediating IDDM. This is supported by animal studies showing that administration of cytokines that promote Th1 development exacerbates the development of diabetes and that monoclonal antibodies specific for Th1-derived cytokines block the development of the disease (Rabinovitch, 1994). In addition, murine β cell–specific T cell clones that exhibit a Th1 phenotype can efficiently transfer disease in syngeneic young NOD recipients (Haskins and McDuffie, 1990; Shimizu et al., 1993; Katz et al., 1995). Th2 cells, which are characterized by the secretion of IL-4, IL-5, IL-6, IL-10, and IL-13 and primarily support humoral mediated immunity, appear to have a down-regulatory role in IDDM. Administration of IL-4 (Rapoport et al., 1993) or IL-10 (Pennline et al., 1994), both of which promote Th2 development and function, protects NOD mice from diabetes. In addition, purified T cells with a CD45RC^{lo} (Th2-like) phenotype prevent an induced form of diabetes in rats (Fowell and Mason, 1993).

Several studies indicate that a functional imbalance between the two Th cell subsets is a key determinant in establishing islet pathology. A high ratio of IFNγ/IL-4 producing T cells can normally be detected in infiltrates leading to the destruction of islets grafted under the kidney capsule in NOD mice (Shehadeh et al., 1993). In contrast, grafted islets in NOD mice containing infiltrates with a lower ratio of IFNγ/IL-4 producing T cells (as a result of receiving Freund's adjuvant) remain intact. Furthermore, a recent study has suggested that an inverse relationship exists between humoral reactivity to GAD and risk for IDDM in prediabetic patient populations (Harrison et al., 1993).

The events that modulate the balance between the two Th subsets in IDDM are still a matter of speculation. Factors that may have quantitative or qualitative effects on T cell activation such as the density of MHC/peptide complexes on the surface of APCs (Pfeiffer et al., 1995), TCR affinity/avidity for the binary complex, or interactions between costimulatory molecules (Lenschow et al., 1995) may lead to preferential development of Th1 cells in IDDM. It is also conceivable that one or more of the several non-MHC genes that confer IDDM susceptibility may be associated with some aspect of Th cell subset development (Scott et al., 1994).

To view the regulation of the disease process strictly in terms of Th1 and Th2 subsets is undoubtly an oversimplification. For example, CD4+ Th1 autoreactive T cell clones have been established from NOD mice that secrete an unknown factor which can suppress the adoptive transfer of diabetes (Akhtar et al., 1995). In addition, T cells expressing a diabetogenic TCR and cultured under conditions to promote Th2 development are unable to mediate protection in NOD recipients (Katz et al., 1995). CD8+ T cells have also been shown to exhibit Th1- and Th2-like phenotypes, and the contribution of cytokines secreted by non–T cells must certainly be considered. The development of a given Th cell subset and, in turn, the outcome of the diabetogenic response undoubtedly involve the interplay of a number of cell types and factors.

Immunotherapy

Early attempts to prevent IDDM typically relied on immunosuppressive drugs (cyclosporine) or drugs that indiscriminantly inhibit cell proliferation (imuran), often leading to serious side effects. Therefore, a great deal of

effort has focused on selectively targeting those T cells involved in the disease process. One general approach has been to employ monoclonal antibodies specific for molecules expressed by the effector T cell population. Monoclonal antibodies specific for CD4 (Shizuru et al., 1988) and CD3, a component of TCRs (Chatenoud et al., 1993), have been shown to be effective in the prevention and treatment, respectively, of diabetes in NOD mice. Similarly, prediabetic NOD mice are protected from disease when treated with antibodies that interfere with antigen recognition (anti-class II, Boitard et al., 1988; anti-TCR, Sempe et al., 1991), cellular activation (anti-B7; Lenschow et al., 1995), and homing to the pancreas (anti-L selectin and anti-VLA-4; Yang et al., 1993). Finally, antibodies targeting cytokines associated with Th1 activity (anti-IFN-γ, anti-TNFα, and anti-IL-12; Rabinovitch, 1994) have been able to prevent disease in prediabetic NOD mice. In general, however, the applicability of antibodies specific for these "immune-related molecules" to human IDDM is limited by the side effects of chronic administration, such as immunogenicity, and the lack of selectivity.

An alternative approach is to devise protocols in which immunomodulation can be selectively applied through the use of a specific antigen/peptide. Recently, it has been demonstrated that insulin, when administered prior to the onset of diabetes, can delay or prevent disease in individuals at high risk for IDDM (Keller et al., 1993). The precise mechanism by which protection is mediated is not known. Both metabolic and immunologic factors may contribute to the effectiveness of this form of therapy. Nevertheless, multicenter trials of subcutaneous insulin prophylaxis to individuals at high risk for developing diabetes have recently been initiated.

In general, antigen-specific tolerance can be induced via two distinct processes: clonal deletion/anergy and induction of regulatory T cells. Clonal deletion/anergy has been shown to be effective in acute experimental autoimmune diseases where the inciting autoantigen/peptide is known. However, the high degree of specificity associated with this approach might be limiting in IDDM, in which the inciting autoantigen is not known, and where spreading of the autoimmune response to a number of epitopes within a single autoantigen and targeting of other autoantigens occur. Despite these reservations, administration of GAD, insulin, or HSP60 (but not carboxypeptidase H or peripherin) to NOD mice appears to result in the induction of antigen-specific regulatory T cells (Th2) that effectively suppress the disease. These regulatory T cells are thought to suppress the effects of nearby diabetogenic T cells through the antigen-stimulated secretion of IL-4, IL-10, and TGFβ. The advantage of this approach is that knowledge of the inciting β cell autoantigen (if only one such antigen truly exists) is not required. However, it is still unclear whether regimens can be devised that effectively induce a long lasting form of active suppression with no deleterious side effects in a clinical setting. For example, oral administration of antigen appears to be nontoxic, but its effects are variable and dose specific. This does not appear to be the case with systemically administered antigen. However, the possibility exists that systemic administration of antigen might have an immunizing effect and exacerbate disease.

Although antigen-specific immunotherapy appears to be a promising method to prevent IDDM, it is most likely that a combination of approaches may prove to be more generally effective. Thus, active suppression by antigen-induced regulatory T cells may be enhanced in concert with antibodies targeting cytokines required for Th1 development and function. Furthermore, as additional β cell autoantigens are identified and shown to have a role in the disease process, therapy might employ a number of autoantigens to target the polyclonal population of autoreactive T cells, thereby increasing the likelihood of successful treatment.

Even if safe, effective, and long lasting immunotherapies are developed, their application is a formidable challenge. Only 15% of new cases of IDDM occur in families with a previous case in the kindred. Overt diabetes develops only when β cell destruction is nearly complete, and the patient is asymptomatic for months or years until that point is reached. Immunotherapy thus must be preventive, which requires inexpensive, accurate genetic, autoantibody, and T cell screening techniques. Given the large number of islet cell autoantigens now available and the rapid progress in identifying genetic susceptibility markers, such screening techniques should soon be feasible. Hopefully, effective methods of prevention will promote widespread population screening and the application of preventive therapy.

References

Ache-Orbea, H., and McDevitt, H.O. (1987). The first external domain of the non-obese diabetic mouse class II I-Aβ chain is unique. Proc. Natl. Acad. Sci. USA *84*, 2435–2439.

Akhtar, I., Gold, J.P., Pan, L.Y., Ferrara, J.L., Yang, X.D., Kim, J.I., and Tan, K.N. (1995). CD4$^+$ β islet cell reactive T cell clones that suppress autoimmune diabetes in nonobese diabetic mice. J. Exp. Med. *182*, 87–97.

Atkinson, M.A., and Maclaren, N.K. (1993). Islet cell autoantigens in insulin-dependent diabetes. J. Clin. Invest. *92*, 1608–1616.

Atkinson, M.A., Bowman, M.A., Campbell, L., Darrow, B.L., Kaufman, D.L., and Maclaren, N.K. (1994). Cellular immunity to a determinant common to glutamic acid decarboxylase and Coxsackie virus in insulin dependent diabetes. J. Clin. Invest. *94*, 2125–2129.

Baekkeskov, S., Aanstoot, H.J., Christgau, S., Reetz, A., Solimena, M., Cascalho, M., Folli, F., Richter-Olesen, W., and de Camilli, P. (1990). Identification of the 64K autoantigen in insulin dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature *347*, 151–156.

Barnett, A.H., Eff, C., Leslie, R.D.G., and Pyke, D.A. (1981). Diabetes in identical twins: a study of 200 pairs. Diabetologia *20*, 404–409.

Boitard, C., Bendelac, A., Richard, M.F., Carnaud, C., and Bach, J.F. (1988). Prevention of diabetes in nonobese diabetic mice by anti-I-A monoclonal antibodies: transfer of protection by splenic T cells. Proc. Natl. Acad. Sci. USA *85*, 9719–9723.

Castano, L., and Eisenbarth, G.S. (1990). Type I diabetes: a chronic autoimmune disease of human, mouse, and rat. Annu. Rev. Immunol. *8*, 647–680.

Chatenoud, L., Thervet, E., Primo, J., and Bach, J.F. (1993). Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. Proc. Natl. Acad. Sci. USA *91*, 123–127.

Christie, M.R., Genovese, S., Cassidy, D., Bosi, E., Brown, T.J., Lai, M., Bonifacio, E., and Bottazzo, G.F. (1994). Antibodies to islet 37K antigen, but not to glutamic acid decarboxylase, discriminate rapid progression to IDDM in endocrine autoimmunity. Diabetes *43*, 1254–1259.

Conrad, B., Weidmann, E., Trucco, G., Rudert, W.A., Behboo, R., Ricordi, C., Rodriguez-Rilo, H., Finegold, D., and Trucco, M. (1994).

Evidence for superantigen involvement in insulin dependent diabetes mellitus aetiology. Nature *371*, 351–355.

Cucca, F., Lampis, R., Frau, F., Macis, D., Angius, E., Masile, P., Chessa, M., Frongia, P., Silvetti, M., Cao, A., DeVirgiliis, S., and Congia, M. (1995). The distribution of DR4 haplotypes in Sardinia suggests a primary association of type I diabetes with DRB1 and DQB1 loci. Human Immunol. *43*, 301–308.

Daniel, D., Gill, R.G., Schloot, N., and Wegman, D. (1995). Epitopa specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice. Eur. J. Immunol. *25*, 1056–1062.

Elias, D., and Cohen, I.R. (1994). Peptide therapy for diabetes in NOD mice. Lancet *343*, 704–706.

Elias, D., Reshef, T., Birk, O.S., van der Zee, R., Walker, M.D., and Cohen, I.R. (1991). Vaccination against autoimmune mouse diabetes with a T cell epitope of the human 65-kDa heat shock protein. Proc. Natl. Acad. Sci. USA *88*, 3088–3091.

Elliott, J.F., Qin, H.Y., Bhatti, S., Smith, D.K., Singh, R.K., Fraga, E., Dillon, T., Lauzon, J., and Singh, B. (1994). Immunization with the larger isoform of mouse glutamic acid decarboxylase (GAD67) prevents autoimmune diabetes in NOD mice. Diabetes *43*, 1494–1499.

Erlich, H.A., Zeidler, A., Chang, J., Shaw, S., Raffel, L.J., Klitz, W., Beshkov, Y., Costin, G., Pressman, S., and Bugawan, T. (1993). HLA class II alleles and susceptibility and resistance to insulin dependent diabetes mellitus in Mexican-American families. Nature Genetics *3*, 358–363.

Fowell, D., and Mason, D. (1993). Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the $CD4^+$ T cell subset that inhibits this autoimmune potential. J. Exp. Med. *177*, 627–636.

Hagopian, W.A., Karlsen, A.E., Gottsater, A., Landin-Olsson, M., Grubin, C.E., Sundkvist, G., Petersen, J.S., Boel, E., Dyrberg, T., and Lernmark, A. (1993). Quantitative assay using recombinant human islet glutamic acid decarboxylase (GAD65) shows that 64K autoantibody positivity at onset predicts diabetes type I. J. Clin. Invest. *91*, 368–374.

Harrison, L.C., Honeyman, M.C., de Alzpurua, H.J., Schmidli, R.S., Colman, P.G., Tait, B.D., and Cram, D.S. (1993). Inverse relation between humoral and cellular immunity to glutamic acid decarboxylase in subjects at risk of insulin dependent diabetes. Lancet *341*, 1365–1369.

Haskins, K., and McDuffie, M. (1990). Acceleration of diabetes in young NOD mice with a $CD4^+$ islet-specific T cell clone. Science *249*, 1433–1436.

Ikegami, H., Kawaguchi, Y., Ueda, H., Fukeda, M., Takakewa, K., Fujioka, Y., Fujisawa, T., Uchida, K., and Ogihara, T. (1993). MHC linked diabetogenic gene of the NOD mouse: molecular mapping of the 3' boundary of the diabetogenic region. Biochem. Biophys. Res. Commun. *192*, 877–882.

Jarpe, A.J., Hickman, M.R., Anderson, J.T., Winter, W.E., and Peck, A.D. (1991). Flow cytometric enumeration of mononuclear cell populations infiltrating the islets of Langerhans in prediabetic NOD mice: development of a model of autoimmune insulitis for type I diabetes. Reg. Immunol. *3*, 305–317.

Katz, J., Benoist, C., and Mathis, D. (1993a). Major histocompatibility complex class I molecules are required for the development of insulitis in nonobese diabetic mice. Eur. J. Immunol. *23*, 3358–3360.

Katz, J.D., Wang, B., Haskins, K., Benoist, C., and Mathis, D. (1993b). Following a diabetogenic T cell from genesis through pathogenesis. Cell *74*, 1089–1100.

Katz, J.D., Benoist, C., and Mathis, D. (1995). T helper cell subsets in insulin dependent diabetes. Science *268*, 1185–1188.

Kaufman, D.L., Clare-Salzler, M., Tian, J., Forsthuber, T., Ting, G.S., Robinson, P., Atkinson, M.A., Sercarz, E.E., Tobin, A.J., and Lehmenn, P.V. (1993). Spontaneous loss of T cell tolerance to glutamic acid decarboxylase in murine insulin dependent diabetes. Nature *366*, 69–72.

Keller, R.J., Eisenbarth, G.S., and Jackson, R.A. (1993). Insulin prophylaxis in individuals at high risk of type I diabetes. Lancet *341*, 927–928.

Larger, E., Becourt, C., Bach, J.F., and Boitard, C. (1995). Pancreatic islet β cells drive T cell-immune responses in the nonobese diabetic mouse model. J. Exp. Med. *181*, 1635–1642.

Lenschow, D.J., Ho, S.C., Sattar, H., Rhee, L., Gray, G., Nabavi, N., Herold, K.C., and Bluestone, J.A. (1995). Differential effects of anti-B7-1 and anti-B7-2 monoclonal treatment on the development of diabetes in the nonobese diabetic mouse. J. Exp. Med. *181*, 1145–1155.

Muir, A., Peck, A., Clare-Salzler, M., Song, Y.H., Cornelius, J., Luchetta, R., Krischer, J., and Maclaren, N. (1995). Insulin immunization of nonobese diabetic mice induces a protective insulitis characterized by diminished intrainsletinterferon-γ transcription. J. Clin. Invest. *95*, 628–634.

Nepom, G.T. (1990). A unified hypothesis for the complex genetics of HLA associations with IDDM. Diabetes *39*, 1153–1157.

Nepom, G.T., and Erlich, H. (1991). MHC class II molecules and autoimmunity. Annu. Rev. Immunol. *9*, 493–525.

Passini, N., Lariçan, J.D., Genovese, S., Appella, E., Sinigaglia, F., and Rogge, L. (1995). The 37/40 kilodalton autoantigen in insulin dependent diabetes mellitus is the putative tyrosine phosphatase IA-2. Proc. Natl. Acad. Sci. USA *92*, 9412–9416.

Pennline, K.J., Roque-Gaffney, E., and Monahan, M. (1994). Recombinant human IL-10 (rHUIL-10) prevents the onset of diabetes in the nonobese diabetic (NOD) mouse. Clin. Immunol. Immunopathol. *71*, 169–176.

Pfeiffer, C., Stein, J., Southwood, S., Ketelaar, H., Sette, A., and Bottomly, K. (1995). Altered peptide ligands can control CD 4 T lymphocyte differentiation in vivo. J. Exp. Med. *181*, 1569–1574.

Pietropaolo, M., Castano, L., Babu, S., Buelow, R., Kuo, Y.L., Martin, S., Martin, A., Powers, A.C., Prochazka, M., Naggert, J., Leiter, E.H., and Eisenbarth, G.S. (1993). Islet cell autoantigen 69 kD (ICA69). Molecular cloning and characterization of a novel diabetes-associated autoantigen. J. Clin. Invest. *92*, 359–371.

Quartey-Papafio, R., Lund, T., Chandler, P., Picard, J., Ozogbe, P., Day, S., Hutchings, P.R., O'Reilly, L., Kioussis, D., Simpson, E., and Cooke, A. (1995). Aspartate at position 57 of nonobese diabetic I-$A^β$ b-chain diminishes the spontaneous incidence of insulin-dependent diabetes mellitus. J. Immunol. *154*, 5567–5575.

Rabinovitch, A. (1994). Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Therapeutic intervention by immunostimulation. Diabetes *43*, 613–621.

Ramensee, H.C., Frieda, T., and Stevanovic, S. (1995). MHC ligands and peptide motifs: first listing. Immunogenetics *41*, 178–228.

Rapoport, M.J., Jaramillo, A., Zipris, D., Lazarus, A.H., Serreze, D.V., Leiter, E.H., Cyopick, P., Danska, J.S., and Delovitch, T.L. (1993). Il-4 reverses T cell proliferation unresponsiveness and prevents the onset of diabetes in NOD mice. J. Exp. Med. *178*, 87–99.

Reich, E.P., von Grafenstein, H., Barlow, A., Swenson, K.E., Williams, K., and Janeway, C.A. (1994). Self peptides isolated from MHC glycoproteins of non-obese diabetic mice. J. Immunol. *152*, 2279–2288.

Roep, B.O., Arden, S.D., de Vries, R.R., and Hutton, J.C. (1990). T cell clones from a type I diabetes patient respond to insulin secretory granule proteins. Nature *345*, 632–634.

Scott, B., Liblau, R., Degermann, S., Marconi, L.A., Ogata, L., Caton, A.J., McDevitt, H.O., and Lo, D. (1994). A role for non-MHC genetic polymorphism in susceptibility to spontaneous autoimmunity. Immunity *1*, 73–82.

Sempe, P., Bedossa, P., Richard, M.F., Vila, M.C., Bach, J.F., and Boitard, C. (1991). Anti-α/β T cell receptor monoclonal antibody provides an efficient therapy for autoimmune diabetes in nonobese diabetic (NOD) mice. Eur. J. Immunol. *21*, 1163–1169.

Serreze, D.V., Leiter, E.H., Christianson, G.J., Greiner, D., and Roopenian, D.C. (1994). Major histocompatibility complex I deficient NOD-B2m null mice are diabetes and insulitis resistant. Diabetes *43*, 505–509.

Shehadeh, N.N., Larosa, F., and Lafferty, K.J. (1993). Altered cytokine activity in adjuvant inhibition of autoimmune diabetes. J. Autoimmun. *6*, 291–300.

Shimizu, J., Kanagawa, O., and Unanue, E.R. (1993). Presentation of β-cell antigens to CD4+ and CD8+ T cells in nonobese diabetic mice. J. Immunol. *151*, 1723–1730.

Shizuru, J.A., Taylor-Edwards, C., Banks, B.A., Gregory, A.K., and Fathman, C.G. (1988). Immunotherapy of the nonobese diabetic mouse: treatment with an antibody to T helper lymphocytes. Science *240*, 659–662.

Singer, S.M., Tisch, R., Yang, X.-D., and McDevitt, H.O. (1993). An $A\beta^d$ transgene prevents diabetes in nonobese diabetic mice by inducing regulatory T cells. Proc. Natl. Acad. Sci. USA *90*, 9566–9595.

Stern, L.J., Brown, J.H., Jardetzky, T.H., Urban, R., Strominger, J.L., and Wiley, D.C. (1994). Crystal structure of the human class II MHC protein HLA DR1 complexed with an influenza virus peptide. Nature London *368*, 215–223.

Thai, A.C., and Eisenbarth, G.S. (1993). Natural history of IDDM. Diabetes Rev. *123*, 37–64.

Tisch, R., Yang, X.D., Singer, S.M., Liblau, R.S., Fugger, L., and McDevitt, H.O. (1993). Immune response to glutamic acid decarboxylase correlates with insulitis in nonobese diabetic mice. Nature *366*, 72–75.

Todd, J., Bell, J., and McDevitt, H.O. (1987). HLA-DQ β gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. Nature *328*, 599–604.

Vyse, T.J., and Todd, J.A. (1996). Genetic analysis of autoimmune disease. Cell, this issue.

Wicker, L.S., Appel, M.C., Dotta, F., Pressey, A., Miller, B.J., DeLarato, N.H., Fischer, P.A., Boltz, R.C., and Peterson, L.B. (1992). Autoimmune syndromes in major histocompatibility complex (MHC) congenic strains of nonobese diabetic (NOD) mice. The MHC is dominant for insulitis and cyclophosphamide-induced diabetes. J. Exp. Med. *176*, 67–77.

Wicker, L.S., Leiter, E.H., Todd, J.A., Renjilian, R.J., Peterson, E., Fischer, P.A., Podolin, P.L., Zijlstra, M., Jaenisch, R., and Peterson, L.B. (1994). b2-microglobulin deficient NOD mice do not develop insulitis or diabetes. Diabetes *43*, 500–504.

Wicker, L.S., Todd, J.A., and Peterson, L.B. (1995). Genetic control of autoimmune diabetes in the NOD mouse. Annu. Rev. Immunol. *13*, 179–200.

Wucherpfennig, K.W., and Strominger, J.L. (1995). Selective binding of self peptides to disease-associated MHC molecules: a mechanism for MHC linked susceptibility to human autoimmune disease. J. Exp. Med. *181*, 1597–1601.

Yang, X.D., Karin, N., Tisch, R., Steinman, L., and McDevitt, H.O. (1993). Inhibition of insulitis and prevention of diabetes in nonobese diabetic mice by blocking L-selectin and very late antigen 4 adhesion receptors. Proc. Natl. Acad. Sci. USA *90*, 10494–10498.

Zhang, Z.J., Davidson, L., Eisenbarth, G., and Weiner, H.L. (1991). Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. Proc. Natl. Acad. Sci. USA *88*, 10252–10256.

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Insulin-Dependent Diabetes Mellitus as an Autoimmune Disease

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I. Introduction

DIABETES mellitus is simply defined on the basis of hyperglycemia. It is, however, a highly heterogeneous disease. A major advance was made in the late 1960s when insulin-dependent diabetes mellitus (IDDM, type 1) was distinguished from non-insulin-dependent diabetes mellitus (NIDDM, type 2). Another milestone was the realization in the 1970s that in most cases IDDM has, presumably, an autoimmune origin (1–4). This offered new clues to the etiology and elicited hopes of immunoprevention, which is still the ultimate goal of research in the immunology of IDDM.

This review will attempt to cover the major pending questions on the origin of the autoimmune process that leads to IDDM and will discuss in some depth genetic predisposition and environmental factors, the interaction of which creates the conditions required for disease onset. This will be followed by a characterization of the anti-β-cell immune response and the mechanisms by which the β-cell lesion is induced. Also discussed will be how physiological tolerance to self-antigens of β-cells is lost in diabetic subjects, as it is the pathogenic event underlying T cell-mediated β-cell aggression. The review will conclude with present and potential clinical applications of these concepts, which have already changed the face of diabetology and will continue to gain momentum. Animal models of the disease will be presented first and will figure strongly throughout this review, inasmuch as they have provided exceptional means for genetic and immunological manipulations inaccessible in man.

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II. Animal Models of IDDM

A broad spectrum of animal models of IDDM have become available over the last 10 yr. They comprise spontaneous models, in which disease develops unprovoked, and experimental models induced by various types of intervention.

A. Spontaneous models

Two major models of IDDM are used: the nonobese diabetic (NOD) mouse and the Bio Breeding (BB) rat, which develop a disease very similar, by most evaluable criteria, to human IDDM.

1. The NOD mouse. The NOD mouse was discovered in Japan in the late 1970s (5). It was inbred, distributed worldwide, and used to establish numerous colonies. These colonies differ widely in the frequency and the age of onset of IDDM (6), owing to multiple environmental factors (see below). Diabetes usually appears between 4 and 6 months of age, much more frequently in females than in males. Clinical diabetes is preceded by infiltration of the pancreatic islets by mononuclear cells (insulitis), which occurs at about 1 month of age in both sexes. In addition to diabetes, NOD mice present thyroiditis (7), sialitis, and, late in life, autoimmune hemolytic anemia (8). Extrapancreatic autoimmune manifestations, including thyroiditis, are also found in a subset of human diabetics with female preponderance (sometimes called type 1b). Recently, interesting new experimental tools have been constructed. They include the NOD/nude mouse, where the nude (athymic) genotype has been introduced by repeated backcrosses in the NOD mouse background (9), and the NOD/scid mouse, in which a mutant gene encoding a defect common to both site-specific DNA recombinational and DNA repair pathways has been introduced into the NOD genome, leading to a severe combined immunodeficiency (10). These models can be used to perform unique experiments of cell transfer without interference from the (deficient) recipient immune system. Also noteworthy is a model of accelerated diabetes induced by cyclophosphamide, an alkylating agent widely used as an immunosuppressive drug. Two injections of 200 mg/kg at a 14-day interval induce diabetes in most male and female mice within 2 to 3 weeks (11, 12) through a mechanism probably involving elimination of regulatory T cells (discussed below).

2. The BB rat. The BB rat was initially developed in Canada in the early 1970s (13). At about 4 months of age it develops severe diabetes, preceded, as in the NOD mouse, by insulitis. A particular feature of the BB rat is the presence, early in life, of major lymphocytopenia (14), involving a particular lymphocyte subset characterized by the RT6 antigen (15). Diabetes is usually associated with thyroiditis in this model. Not all BB rats develop diabetes: a subset of BB rats representing a genetic drift are diabetes resistant (DR-BB).

B. Experimentally induced diabetes

At variance with most autoimmune diseases, in which the target autoantigens are known, we as yet have no experimental model of diabetes induced by administration of the target β-cell autoantigen incorporated in adjuvant, with the exception of transient diabetes induced by a peptide derived from a candidate target autoantigen, heat shock protein 60 (16, 17). Fortunately, numerous other experimental models are available.

1. Chemically induced diabetes. Streptozotocin (STZ)-induced diabetes. β-Cell destruction can be achieved by administering high doses of β-cell-selective toxic agents such as STZ (18) and alloxan (19). Repeated administration of STZ at low, subdiabetogenic doses also causes diabetes preceded by insulitis (20). Such low dose STZ-induced diabetes appears to be immunologically mediated, as indicated by resistance of athymic mice (21) and prevention by immunosuppressive agents (20) even if some intriguing data have recently been reported showing induction of the low-dose STZ diabetes in NOD-scid/scid mice in the absence of functional lymphocytes (21a). The mechanisms of insulitis and diabetes appear to relate to STZ-induced changes in islet immunogenicity: insulitis only appears on islets grafted in STZ-treated mice if grafting is performed before STZ administration or if the islets are first exposed to STZ *in vitro* (22). The mechanisms of these changes are not fully understood but might be related to the induction by STZ of increased expression of class II molecules of the major histocompatibility complex (MHC) on β-cells. This increased expression has been directly visualized (23), and low-dose STZ-induced diabetes is prevented by anti-interferon-γ (IFNγ) antibody therapy (24), which is known to inhibit MHC molecule expression. The relevance of this mechanism to human IDDM pathogenesis will be discussed later, but it is interesting to note here that NOD mice are susceptible to lower repeated STZ doses than conventional strains with the highest STZ sensitivity (25, 26), pointing to the possible role of toxic environmental factors in genetically predisposed individuals.

2. Immunomanipulation. Thymectomy performed within 2 days after birth can induce a flourishing state of autoimmunity in mice (27). Whether the emergence of autoreactive clones is due to elimination of the censor function of the thymus (negative selection of autoreactive clones) or to the loss of suppressor function is still being debated (27). Similarly, insulitis and diabetes (associated with thyroiditis) can be induced in normal non-autoimmune adult rats by combining adult thymectomy and sublethal irradiation (28, 29) or in athymic rats by transfer of normal spleen cells (30). The disease can be prevented by administration of CD4+RT6+ T cells derived from normal rats (28) or facilitated in the adoptive transfer model by prior depletion of RT6+ cells *in vivo* (30), suggesting that in both models diabetes is due to the elimination of a RT6+ T cell subset with suppressor function. It is interesting to note the paradox between these models in which thymectomy promotes diabetes and the observation, discussed later, that neonatal thymectomy prevents the onset of diabetes in NOD mice and BB rats. One may presume that in the latter case thymectomy prevents the differentiation of effector T cells (perhaps together with that of helper T cells) while in the former, where thymectomy is slightly delayed, there is only inhibition of suppressor T cell differentiation.

3. *Transgenic mice.* Selective expression of various transgenes in β-cells can be induced by coupling them to the insulin gene promoter. This strategy has been applied successfully to a number of models, leading to the induction of insulitis and/or diabetes. Insulitis, the hallmark of immunologically mediated diabetes, can be induced in mice transgenic for the simian virus SV40 T antigen gene when the transgene is expressed in β-cells late in ontogeny (after thymic negative selection has taken place) (31). Insulitis is the consequence of an anti-T antigen T cell-mediated response. Interestingly, when the T antigen is expressed earlier in ontogeny, mice are tolerant to the antigen and do not become diabetic (but they may then develop insulinoma). Similar results can be obtained with the IFNγ gene, which probably operates by enhancing the expression of class II MHC molecules in β-cells (32). Diabetes in such transgenic mice is of an autoimmune nature, since the disease is transferred to normal syngeneic islets grafted into the transgenic mice, and lymphoid cells from the transgenic mice are cytotoxic to normal islets *in vitro* (33). Similar but less clear-cut data have been reported with IFNα (34), tumor necrosis factor-α (TNFα) (35, 36), and interleukin (IL)-10 (37). Interestingly, in the two latter cases insulitis occurred but diabetes did not (*i.e.* there was no β-cell lysis).

Another approach consists of expressing various genes, notably viral genes, early enough in development to prevent anti-β-cell sensitization and then attempting to provoke IDDM either by infecting the mice with the corresponding virus or by hybridizing them with other transgenic mice expressing the genes for T cell receptors (TCR) specific to the transgene-encoded antigen. Oldstone *et al.* (38) showed that transgenic mice expressing the gene of the murine lymphocytic choriomeningitis virus (LCMV) glycoprotein became diabetic after infection with LCMV, due to destruction by antigen-specific cytotoxic T cells of β-cells expressing the viral antigen. This shows that selective expression of the viral antigen in β-cells (and presumably not in the thymus) early in development does not lead to tolerance toward this antigen, since it would have then prevented sensitization in the adult. Insulitis and diabetes have been observed in the absence of viral infection in transgenic mice expressing the influenza virus hemagglutinin in β-cells (39). One should note, however, that in a similar model Lo *et al.* (40) failed to induce diabetes in transgenic mice expressing the influenza virus hemagglutinin in β-cells even after infection with hemagglutinin-expressing viruses.

The double transgenic strategy has been used successfully for the influenza virus hemagglutinin (40a) and the glycoprotein of LCMV (41). It is important to note that infection by the virus was necessary to obtain diabetes in the case of LCMV glycoprotein, suggesting that in certain experimental conditions nontolerant T cells may ignore their target antigens expressed in β-cells. Viral infection may then stimulate the recognition of the antigen and T cell activation, indicating that overcoming ignorance may require T cell preactivation. Virus infection was not required to obtain diabetes in the influenza hemagglutinin model. This difference suggests that, depending on the transgenic mice utilized (*i.e.* MHC and non-MHC genotype, MHC class I or class II restriction, environmental factors, etc.), coexpression of the target antigen in β-cells and the corresponding TCR is sufficient for diabetes to occur in the absence of T cell activation. We shall see below that NOD mice transgenic for the TCR of diabetogenic T cell clones develop accelerated diabetes. However, it should be emphasized that coexistence of the antigen and the specific TCR does not necessarily lead to elimination or activation of these T cells suggests that, in these settings, T cells may "ignore" their target antigen.

Diabetes can also occur in transgenic mice expressing MHC class I (42) or class II (43–44) genes in β-cells; however, in this case diabetes is not due to an immune reaction (*i.e.* there is no insulitis) but rather to β-cell functional alterations due to overexpression of multiple copies of the MHC molecules. Indeed, β-cell expression of smaller amounts of MHC molecules does not induce diabetes (45), and expression of non-MHC molecules such as calmodulin can induce a similar type of nonimmune IDDM (46).

TABLE 1. Transgenic mice for the study of IDDM

Immune diabetes or insulitis.		
Single transgenics (transgene coupled to insulin promoter)		
SV40-T antigen (31)		
IFN-γ (32, 33, 34)		
TNF (35, 36)		
IL-10 (37)		
LCMV glycoprotein + virus infection (38, 242)		
Influenza virus hemagglutinin (39)		
Double transgenics		
Influenza virus hemagglutinin (β-cells) + TCR (40a)		
LCMV + TCR + virus infection (β-cells) (41)		
Nonimmune IDDM (without insulitis)		
MHC class I (42)		
MHC class II (43, 44)		
Calmodulin (46)		
Transgenic NOD mice (protection from insulitis and/or diabetes)		
I-A	$I\text{-}A^d$	(257)
	$I\text{-}A^k$	(53, 54, 256)
	Aα (Pro 56)	(55)
I-E	E^{C57BL}	(56)
	$E\alpha^d$	(55)
	$E\alpha^k$	(57)
L^d		(58)

C. Lessons from animal models

These animal models have enabled us to make remarkable progress over the last few years in understanding the pathogenesis of IDDM. They have been used for the transfer experiments necessary to prove the autoimmune nature of the disease and have allowed the production of islet-specific T cell clones and enabled fine analysis of MHC and non-MHC diabetes predisposing genes. Finally, they have allowed the evaluation of the various immunointervention procedures to be potentially used in man.

It is essential to bear in mind, however, that IDDM is a heterogeneous disease (see *Section IX.A*) and that each animal model represents, at best, the counterpart of an individual

human case reproduced in multiple copies. In addition, it should be remembered that most experimentally induced models correspond to highly artificial situations far from the conditions in which spontaneous disease develops.

III. Genetics of IDDM

A. Introduction: familial transmission of the disease

IDDM has long been known as a hereditary disease on the basis of the relatively high rate of familial transmission: the risk of becoming diabetic is approximately 7% for a sibling and 6% for a child of a diabetic (47). The disease concordance rate is approximately 35–40% in identical twins (47, 48) but penetrance of genetic factors evaluated from the identical twin concordance rate is probably less than 40% for the following reasons: 1) twins share more environmental factors than unrelated individuals, 2) there is a tendency for disease-concordant identical twins to respond more to population calls than nonconcordant twins, and 3) a significant percentage of twins carrying the whole set of predisposing genes are both resistant to the disease.

B. Approaches to identifying IDDM predisposition genes

The above patterns of familial transmission, combined with data from animal models, indicate that the determinism of IDDM is polygenic and multifactorial. The search for predisposition genes is complex, especially as most if not all predisposition genes appear to be basically "normal" *i.e.* without mutations or deletions. A fortuitous combination of these genes, together with permissive or triggering environmental factors, provokes the disease. Each of these genes may be present in a large proportion of healthy subjects (notably the patient's nondiabetic relatives).

There are two distinct strategies for identifying IDDM predisposition genes. In the first, one selects candidate genes coding for presumed elements of the pathological process such as the T cell receptor, MHC molecules, β-cell autoantigens, insulin, and cytokines and seeks links between their polymorphism and the disease.

In the second approach (identical to that used for monogenic diseases), segregation of the disease or of one of its major traits (partial phenotype) and that of polymorphic markers distributed throughout the genome are studied in parallel in multiplex families. The most easily accessible and informative markers now available are microsatellites, *i.e.* simple sequence repeats whose length varies between individuals in an allele-stable fashion.

C. The role of the MHC

Given the major role of MHC molecules in antigen presentation to T cells, MHC genes are obvious candidate predisposition genes for IDDM (and all other autoimmune diseases), even if, in fact, their association with IDDM was discovered fortuitously (49, 50), before MHC restriction of antigen recognition by T cells was unraveled.

The role of the MHC in genetic predisposition to IDDM is predominant, as shown by the high disease concordance rate in HLA-identical siblings (~12%, and even 15-17% in DR3/4 heterozygotes) (47). It is also fully confirmed in murine models of the disease by segregation studies (51), by the absence of diabetes observed in congenic mice genetically identical to NOD mice except for the MHC (52), and by the prevention of the disease by introduction of various MHC transgenes differing from the NOD MHC, either class II (I-A) (53–55), I-E (55–57), or class I (58).

1. Animal models. Diabetes onset is closely dependent on the MHC in NOD mice at the level of the I-A locus (51, 59) in a dominant fashion (52). Sequence analysis of the I-A^{NOD} gene has shown that this allele has a serine residue at position 57 of the β-chain at variance with all common mouse strains that have an Asp at that position (60). However, absence of Asp at position 59 does not entirely explain the role of the MHC, since transgenic mice expressing I-A genes without an Asp at position 57 of the I-Aβ-chain can be protected from diabetes (54, 55). Also, NOD mice do not express genes of the other I locus, I-E, owing to a mutation of the Eα promoter region (59). NOD mice transgenic for I-E (55–57) are protected from the disease, an important finding suggesting a protective role of I-E genes even though I-E+ NOD mice obtained by backcrossing with I-E+ strains may develop diabetes (61). Segregation studies have also pointed to the major predisposing role of the MHC in the BB rat with partial dominance of the RT-1^u-allele (62). Interestingly, in RT-1^u × RT-1^b crosses, diabetes is associated with the u-allele, whereas thyroiditis is associated with the b-allele (63). It remains to be determined whether class II loci are exclusively involved in the MHC-associated predisposition to diabetes in these animal models (independently from the numerous non-MHC predisposing genes to be discussed below).

2. Human IDDM. IDDM is positively associated in Caucasians with two sets of alleles: 1) HLA A1, B8, DR3 DQB1*0201 and 2) DR4 DQB1*0302 (64–65). This association was initially shown by means of serological typing (49, 50) and has now been confirmed by direct genomic typing with polymerase chain reaction and hybridization using sequence-specific oligonucleotide probes (66, 67). The association holds for alleles on neighboring loci (haplotype) because of the tight linkage disequilibrium in the MHC. This is particularly true for the ancestral extended haplotype A1 B8 DR3 DQB1*0201 DQA1*0501, which comprises class III genes and the TAP2*0101 allele, making it difficult for this haplotype to determine the precise locus that predisposes to the disease. The case is clearer for DR4-DQB1*0302, where the DQ locus seems to be directly involved (64, 65) in keeping with the putative Ir gene function of class II genes (*i.e.* HLA class II molecules bind antigenic peptides and present them as a molecular complex to the TCR). Attention has been drawn to the nature of the residue at position 57 of the HLA DQβ-chain (absence of Asp in IDDM-predisposing alleles) (68). The Asp residue is much more rarely found in diabetics than in the general population and almost never in double copy (homozygous state). This observation is particularly interesting in view of the critical place of this residue in MHC-peptide interactions. The highest relative risk is ob-

served in DR3/4 heterozygotes, with a disease frequency higher than that predicted from the relative risks associated with individual alleles. It is not known whether this apparent synergy is due to the synergistic interaction between two independent HLA genes or to the creation of a hybrid molecule made of chains encoded by the two alleles (65). Such transcomplementation has been formally demonstrated (69) but its pathogenic role is uncertain.

Other MHC genes are associated with IDDM protection, their frequency being lower in diabetics than in the general population. This is the case for DR2 (63, 64, 66, 67) and for the TAP2*0201 allele (70, 71), which codes for a transporter of antigenic peptides to MHC class I molecules. Whether this latter association is intrinsic or relates to a linkage disequilibrium with class II alleles remains to be determined. It is important to continue investigations of the mechanisms of MHC-associated IDDM protection, which could include, in addition to defective peptide transport, peptide capture by the protective HLA molecules that prevents binding of the peptide to the predisposing HLA molecules and, thus, its effective presentation to T cells or the generation of suppressor cells of the TH2 type (63). Finally, note that analysis of the MHC-IDDM association is complicated by disease heterogeneity, notably in terms of age of onset (67) and ethnic origin (63–65).

D. Non-MHC genes

The involvement of non-MHC genes in the predisposition to IDDM is demonstrated by the above mentioned difference in the disease concordance rate in identical twins (35–40%) and HLA-identical siblings (~12%). The search for candidate non-MHC predisposing genes has so far been relatively unfruitful in human IDDM. Nonetheless, the insulin gene has been shown to be associated with IDDM (72–74), particularly in HLA DR4 subjects (73). In the same study, it was shown that the insulin gene effect was stronger in paternal meiosis, suggesting a role for maternal imprinting (72). However, the involvement of these two features (DR4 preference and paternal meiosis) was not confirmed in another study (74). It remains to be shown whether the association relates to the insulin gene itself, as suggested by a recent mapping study (75), or to a neighboring gene. Studies of the polymorphism of another logical candidate gene, TCR, have failed to provide clear-cut results (76–78).

Studies of the NOD mouse have been more fruitful. Segregation studies using microsatellites have led to the description of 12 non-MHC predisposition loci (Refs. 51 and 79–83 and Table 2), in addition to the major association with MHC loci on chromosome 17. One of the genes on chromosome 1 could be bcl2 (80), a proto-oncogene known to have anti-apoptosis functions. Delayed T cell apoptosis, directly demonstrated in NOD mice (84), could favor survival and activation of autoreactive T cells, in keeping with similar data obtained in MRL/l lupus mice showing a mutation of the FAS gene, known for its apoptosis function. One of the genes on chromosome 3 has been narrowed down to the IL-2 gene, which has a different sequence in NOD mice than in common mouse strains, including an insertion and a deletion of tandem repeat sequences that encode amino acid repeats in the mature protein (81). The other gene on chromosome 3 has been mapped to the gene coding for the higher affinity receptor for immunoglobulin G (83). The nature and expression of the other predisposition genes are unknown.

TABLE 2. Genes predisposing to IDDM

		References
NOD mouse		
MHC (ch 17)	I-A	51, 52, 59, 81
	I-E (absence of expression)	59
Ch 1	IL-1R	79
	bcl2	80
Ch 3	IL-2	81
	high affinity Fcγ receptor	51, 81–83
Ch 4		81
Ch 6		51, 81, 82
Ch 7		81
Ch 9		81
Ch 11 (early-onset cytoxan-induced diabetes)		51, 81
Ch 14		81
Ch 15		82
BB rats		
MHC ($RT1^u$)		61
Ch 4 lyp (lymphocytopenia)		85
Man		
MHC		64–68
A1 B8 DR3 DQB 201 DQA 501 TAP2-A		
DR4 DQB 302 DQA 301		
+ protection DR15(2) DQB 602 DQA 102		
Insulin		72–75

Studies in the BB rat have been less informative. They indicate, however, that lymphocytopenia is encoded by the autosomal gene lyp on chromosome 4, close to the neuropeptide Y gene (85).

E. Conclusions

Taken together, these data suggest the existence of stage-specific genetic control of IDDM. bcl2 And other genes could control an intrinsic nonantigen-specific anomaly of T cells, which could explain the initial mononuclear cell infiltration of the islets (periinsulitis) and other organs (*e.g.* sialitis), as well as the association with other autoimmune traits. The MHC would then play the central role in β-cell autoantigen recognition. Other genes are probably involved, such as those coding for immunoregulatory cells (that amplify the autoimmune reaction), notably cytokine genes (*e.g.* the mutant IL-2 gene mentioned above) and genes controlling β-cell sensitivity to the immune aggression. When these genes are identified, the problem will be to determine their relative contribution to genetic predisposition. It may turn out that all susceptibility genes (defined on the basis of segregation studies) are effectively involved in the pathogenic process, but that their contribution to increasing the relative risk may be highly variable; this will depend not only on the importance of their functional role but also on the frequency of the predisposing allele in the general population. The fairly high concordance rate between siblings, despite relatively low penetrance, argues for a small number of major predis-

position genes (MHC plus perhaps two or three non-MHC genes). This does not, however, rule out the involvement of a multitude of minor genes with an accessory pathogenic role (not mandatory), present in a large fraction of the general population or "used" in a very limited number of patients (genetic heterogeneity). It is likely that some of the genes recently identified in the NOD mouse are minor susceptibility genes of these types.

IV. The Role of the Environment: Does it Trigger or Just Modulate the Anti-β-Cell Autoimmune Response?

A. Introduction

1. Evidence for the role of environmental factors. Several lines of evidence point to a major role of environmental factors in the pathogenesis of IDDM. First, more than 60% of identical twins are discordant for the disease, and it is quite unlikely that this is due to differential somatic rearrangement of T cell receptors. Second, disease frequency varies enormously from country to country (86), and these differences cannot simply be explained by ethnic genetic differences since migrants from countries with a low IDDM frequency to countries with a high frequency are more susceptible than their compatriots (87). Intriguingly, northern countries are more exposed to the disease than southern countries (86); it will be critical to discover the factor(s) responsible for this North/South gradient. Third, a number of apparently nonimmunological interventions can increase or decrease the disease rate in animal models: specific diets [low essential fatty acid (88) or protein intake (89, 90)] and several viral infections (91–95) can reduce disease susceptibility in NOD mice and BB rats, while Kilham's virus (96) and cow's milk (97, 98) can increase it in BB rats. These factors, particularly viral infections, probably explain the variations in disease frequency between NOD colonies (6).

Finally, disease incidence is on the increase in most countries [a 2-fold rise has occurred in Finland over the last 15 yr (99)], strongly pointing to an environmental influence; this holds true even in areas with a distinct genetic background such as Sardinia, where the incidence has recently increased dramatically to values much higher than those in surrounding regions (100).

Not only do environmental factors seem to influence IDDM onset, they can also apparently alter the course of the disease. These factors can be shared by the whole population (climatic factors, hygiene, etc.), or by a given family (*e.g.* eating habits), or be specific to the individual (*e.g.* travels and sexual partners). Retrospective epidemiological studies are difficult to interpret, but prospective testing of candidate environmental factors holds out far more promise. Such a study of cow's milk feeding in the first weeks of life is underway.

2. Trigger or modulator? It is generally agreed that environmental factors are at the origin of a large number of diseases. This is certainly the case for infectious diseases, even if the genetic background can strongly influence disease expression. The situation is very different in the case of diseases in which environmental factors essentially modulate the expression of predisposing genes, either positively (predisposing factors) or negatively (protective factors). In the case of triggering factors, disease onset is directly related to the encounter with the environmental factor (usually single and limited in time), which can then be considered as the cause of the disease. In the "modulation" hypothesis, the disease can only appear in the fraction of the population at genetic risk and it is on this population that environmental factors (usually multiple and chronic) exert their positive or negative effect. Available data suggest that IDDM is of the second type.

B. Viruses and IDDM. Interactions with the immune system

A viral origin of IDDM was one of the first etiological hypotheses (101, 102), but the data on which it was based are more complex than initially thought and must now be interpreted in light of data on the autoimmune pathogenesis of the disease. Nonetheless, the viral origin of IDDM remains a central point of debate in the etiology of the disease.

1. Epidemiological data supporting the etiological role of viruses. IDDM onset is often seasonal (103) and could follow outbreaks of certain infections (101, 102). Particular attention has been paid to rubella virus [~ one-third of *in utero* rubella cases develop IDDM (104)] and Coxsackie virus (101, 102, 105, 106). A strain of Coxsackie B virus isolated from a pancreas collected from a single child who died from recent-onset IDDM was able to induce IDDM in mice. It may also be of interest that anti-Coxsackie B virus antibodies have been found in an abnormally high percentage of type 1 diabetics (106).

2. Animal models of virus-induced diabetes. A number of viruses can induce diabetes in various animal species, notably the encephalomyocarditis virus (EMCV), which induces diabetes in several mouse strains (without linkage to the MHC) (107). The effect seems to be mediated by a direct cytolytic effect of the virus, although in the case of some virus variants, diabetes can be prevented by anti-CD4 monoclonal antibodies (108) or irradiation and does not develop in athymic mice (109). This suggests the possibility of an immune phase after the initial direct cytolytic effect of the virus. Other viruses inducing diabetes in animals include reovirus type 1 in mice (with insulitis) (102) and rubella virus in Syrian hamsters (110). Also, it is worth mentioning the endogenous xenotropic retrovirus expression in β-cells of NOD mice (111, 112) and the spectacular triggering of diabetes in the diabetes-resistant DR subline of BB rats after infection by Kilham's virus (96), diabetes apparently caused by a direct cytolytic effect of the virus on β-cells (113).

3. Mechanisms of virus-induced IDDM. Several mechanisms are feasible. The most obvious, clearly demonstrated in several of the models just mentioned (notably EMCV infection in mice and Kilham's virus in DR BB rats), is a direct cytolytic effect of the virus on β-cells. Another mechanism, not exclusive of the first, involves a T cell immune reaction to the virus neoantigens induced at the β-cell surface. This mech-

anism is best illustrated by the model of SV40 transgenic mice expressing the T antigen in β-cells late in ontogeny (31) at a stage of immunological development where exogenous antigens do not induce tolerance. Another possibility is endogenous, vertically transmitted viruses as illustrated by transgenic mice whose β-cells express the LCMV glycoprotein or influenza virus hemagglutinin; these mice become diabetic after viral infection (38, 39) or hybridization with mice transgenic for the antiviral protein TCR (41). It may be worth recalling here that, depending on the virus and (perhaps) the mouse strain, these double transgenic mice require viral infection to become diabetic, suggesting that virus-induced T cell activation may be necessary for diabetes onset, at least in some cases.

Alternative mechanisms are related to molecular mimicry, by which a nontolerized exogenous antigen cross-reacting with a tolerized autoantigen can break down the tolerance to the latter. In the case of IDDM-inducing viruses, virus proteins could conceivably share a sequence with a β-cell autoantigen, as exemplified by the homology between a Coxsackie B viral protein and glutamic acid decarboxylase, a β-cell autoantibody described below (114). Molecular mimicry might also apply to cross-reactivity between an antiviral antibody idiotype and a β-cell autoantigen.

A more trivial interpretation, providing the most likely explanation for the emergence of IDDM after an acute viral infection, is related to the increase in insulin requirements that follows some viral infections: there is no other plausible explanation for the temporal relationship between an acute infection and IDDM onset in most cases, since islet cell autoantibodies are produced several years before the clinical onset of IDDM and consequently long before, not after, the acute infection in question.

4. Viral infections and protection from IDDM in genetically predisposed individuals. Intriguing evidence has recently emerged suggesting that some viruses can protect genetically predisposed animals from diabetes. For example, infection with the mouse lymphocytic choriomeningitis virus (91, 92), the lactodehydrogenase virus (93), or the murine hepatitis virus (94) prevents IDDM in NOD mice when contracted before 2 months of age. These data are in keeping with the observation that both NOD mice (our unpublished data) and BB rats (95) show an increased incidence of the disease when raised in germ-free conditions. The mechanisms of this virus-associated protection are not clear but could involve antigenic competition in the larger sense of the term. For example, viruses could activate the production of immunosuppressive cytokines (of the TH2 type described below). It is important to determine whether the North-South gradient of diabetes incidence mentioned above is partly due to common viral infections; for example, due to the lower temperature and better hygiene, inhabitants of northern countries may be less exposed to infections than those in southern countries, as is the case for hepatitis A virus and cytomegalovirus. This hypothesis is supported by the similar North/South gradient observed for multiple sclerosis, another T cell-mediated autoimmune disease, and the inverse South/North gradient observed for carriage of antibodies to hepatitis A virus used as a marker of infection by water-borne pathogen (95a).

5. Conclusions. It is difficult to unify so diverse and sometimes contradictory data and hypotheses. It can, however, be assumed that some viruses nonspecifically protect against diabetes, while others can induce the disease, either by a direct cytolytic effect or through the T cell response to viral neoantigens expressed at the β-cell surface. In spite of convincing experimental models, however, there is no convincing evidence for a direct pathogenetic role of a virus in human IDDM, at least in the vast majority of cases in which the involvement of the immune system is well documented (see below). In contrast, a chronic viral infection of β-cells is possible, where β-cell neoantigens stimulate a T cell response like that observed in the SV40 transgenic model described above (31). A vertically transmitted virus could also be involved since, as illustrated by the LCMV or influenza virus hemagglutinin transgenic models (38, 39, 41), fetal expression of viral neoantigens by β-cells does not necessarily induce tolerance to the viral antigens. This observation indicates that the immune response to the neoantigen(s) cross-reacts with β-cell autoantigens in uninfected individuals, since diabetes can be transferred to nondiabetic individuals presumably not infected by the virus. Insulitis reappears rapidly in syngeneic pancreas transplants derived from a monozygotic twin placed in a diabetic patient (115). Similarly, IDDM has been described after allogeneic bone marrow transplantation from a diabetic donor (116, 117). These observations are in keeping with those made in NOD mice and BB rats showing recurrence of IDDM when normal allogeneic islets are grafted in conditions avoiding allograft rejection (118, 119). Note, however, that one cannot rule out in all these settings the possible viral contamination of the graft, which casts a doubt on the interpretation of these results.

C. Mycobacteria and IDDM

Freund's complete adjuvant (CFA), which consists of mycobacteria incorporated in a water-in-oil emulsion, completely prevented the onset of IDDM when injected in young NOD mice (120, 121) and BB rats (122). Spleen cells from CFA-protected animals suppress responses of cocultured syngeneic control spleen cells to mitogens *in vitro* (120, 121) and protection can be transferred by spleen cells from the CFA-treated animals to naive animals (123). The nature of the protective cells is still uncertain (macrophages, NK cells, TH2 cells). These data, which have been reproduced with Bacillus-Calmette-Guerin (BCG) vaccine in NOD mice (124), were sufficiently convincing to warrant a therapeutic trial in human prediabetes with BCG (124a).

D. Toxic agents

As mentioned above, several toxic agents show β-cell selectivity and induce IDDM at doses not provoking significant extrapancreatic toxicity (125). This is the case of STZ (18) and alloxan (19). Another agent, Vacor (a rodenticide), has also been shown to induce IDDM at the high doses used in suicide attempts (126). Pentamidine, a drug given to AIDS

patients for prophylaxis of *Pneumocystis carinii* pneumonia, may have a similar effect (127). However, there is little evidence that any toxic agent, whatever its mechanism of action, is at the origin of common forms of IDDM. At most, some toxic agents could act by amplifying the anti-β-cell autoimmune response, as in the low-dose STZ model described above (20), since diabetes onset is accelerated in NOD mice at STZ doses lower than those inducing diabetes in conventional strains (25, 26).

E. Food constituents. The cows' milk hypothesis

Diets are known to influence glucose metabolism, with obvious consequences for diabetics. A number of diets, independent of their direct glycemic effects, have recently been shown to delay the onset of IDDM in NOD mice and BB rats, probably by interfering with the anti-islet immune response. This is the case for low essential fatty acid (89) and protein diets (90).

Conversely, cow's milk accelerates the course of diabetes in BB rats, while lactalbumin-free diets are protective when administered for the first 2 to 3 months of life (97, 98). A role for the whey protein BSA has been suggested, because early induction of tolerance to BSA prevents IDDM and anti-BSA immunization accelerates it in BB rats (see Ref. 128). Much attention has been paid to the possibility that a BSA-related protein could represent an important triggering factor for human IDDM. Anti-BSA antibodies are found in diabetics more frequently than normal (using a particle concentration fluoroimmunoassay) (128, 129). Diabetic children have an abnormally high frequency of immunoglobulin A (IgA) antibodies to β-lactoglobulin (130–132). It is important to mention, however, that these findings are based on a precise methodology and have not always proven easy to repeat (133). Anti-BSA antibodies in diabetics recognize a peptide sequence (ABBOS) containing 17 amino acids in a region of the BSA molecule extending from position 152 to position 168, *i.e.* the site of the major sequence difference with human, mouse, and rat albumin. This peptide sequence cross-reacts with a 69 kilodalton (kDa) β-cell autoantigen (p69), which has recently been cloned independently by two laboratories using anti-BSA (141a) or anti-islet cell antibody-positive diabetics' sera (134) to screen a human pancreas cDNA library. This cross-reaction could explain the stimulation of the anti-islet T cell response by cow's milk in the first week of life (molecular mimicry). It should be noted, however, that at variance with this hypothesis, recent onset diabetics do not show T cell hypersensitivity to BSA or ABBOS (133). Nonrandomized data indicate that exclusive breast-feeding, with delayed exposure to infant formula based on cow's milk, significantly reduces the risk of diabetes in Finnish children (129). A prospective randomized trial has been set up to confirm these data.

F. Stress

There is mounting evidence that psychoaffective events can influence immunity, and some groups have focussed on stress as a possible trigger of IDDM (135–138). It has thus recently been shown that acute stress can accelerate the onset of diabetes in NOD mice (137), whereas raised environmental temperature reduced it (138).

G. Sex hormones

Diabetes is much more common in female than in male NOD mice (5) and its onset is accelerated in males by castration, particularly when combined with thymectomy (139). Conversely, androgen treatment of female mice prevents diabetes (140). The mechanism of action of sex hormones on the immune system is unclear but could involve an effect on immunoregulatory networks: male NOD mice develop insulitis, but most do not become diabetic unless given cyclophosphamide, a drug known to affect suppressor cells (11, 12).

V. Does IDDM Fulfill the Criteria of an Autoimmune Disease?

A. Definition of autoimmune diseases

Autoimmune diseases are diseases due to the pathogenic effect of autoantibodies or autoreactive T cells that provoke inflammation, functional alterations, or anatomical lesions. They must be distinguished from diseases associated with autoimmune manifestations not directly related to disease pathogenesis.

B. Criteria defining autoimmune diseases

Four criteria usually have to be met to consider a disease as autoimmune (141).

1. The disease state can be transferred by the patients' antibodies or T cells.

2. The disease course can be slowed or prevented by immunosuppressive therapy.

3. The disease is associated with manifestations of humoral or cell-mediated autoimmunity directed against the target organ.

4. The disease can be experimentally induced by sensitization against an autoantigen present in the target organ, which presupposes the knowledge of the target autoantigen.

Points 1 and 2 are mandatory. Points 3 and 4 are important but less critical. In fact, only a few so-called autoimmune diseases fulfill all four criteria (one example is myasthenia gravis due to anti-acetylcholine receptor autoantibodies).

C. IDDM as an autoimmune disease

Human IDDM fulfills three of these criteria and indirect arguments exist in animal models for the fourth.

1. Diabetes transfer. Diabetes can be transferred in NOD mice and BB rats into nondiabetic syngeneic animals by spleen cells from diabetic animals (9, 10, 142–144). More precisely, it has been shown using purified T cell preparations and T cell clones derived from spleen or islets of NOD mice that the transfer was exclusively due to T cells (142, 144–146).

We shall see below the phenotype and repertoire of such diabetogenic T cells. Similarly, appearance of diabetes has been observed in man after pancreas transplantation between identical twins (115). Such diabetes is likely due to infiltration of the transplanted pancreas by the recipient autoimmune cells (whether or not they have been reactivated by reexposure to pancreas autoantigen). One should also mention diabetes observed after allogeneic bone marrow transplantation with a diabetic donor (116, 117). The situation is less pure in the latter models since one cannot exclude that non lymphoid cells present in the donor bone marrow could be responsible for the transfer.

2. Effect of immunosuppression. Insulin β-cell damage can be slowed by immunosuppressive therapy, notably cyclosporine (147, 148) and many other immunosuppressive agents essentially active at the T cell level in NOD mice, BB rats (149, Tables 3 and 4), and man (Table 5). The effect is better observed when the treatment is applied early, which is obviously much more difficult to achieve in man than in animal models, but some significant effect is still seen at the disease onset (Table 3).

3. Manifestations of anti-β-cell autoreactivity. There is evidence for both islet-reactive autoantibodies and T cells [*e.g.* islet cell antibodies (ICA) (150), glutamic acid decarboxylase (GAD)-reactive antibodies (151), and T cells (152, 153)].

a. Autoantibodies. Diabetic patients and rodents mount a multifaceted humoral immune response to islet cells. Autoantibodies are found against a wide array of membrane and cytoplasm constituents of β-cells, including insulin (anti-insulin autoantibodies are detected before starting insulin therapy) (154), proinsulin (155), and GAD (151). The most commonly screened antibodies, whose description in 1974 (150) led to the first strong evidence for the autoimmune origin of IDDM, are the so-called ICAs detected by indirect immunofluorescence on human pancreas sections. ICAs bind to the cytoplasm of β-cells [perhaps to gangliosides (156)], but they also usually bind to the cytoplasm of other islet endocrine cells. There are, however, "restricted ICAs" that selectively bind to β-cells (157), which essentially include antibodies directed at GAD (see below). Some interest was initially paid to antibodies directed against islet surface antigens that can be cytotoxic to β-cells (158) or inhibit insulin release by β-cells in the presence of complement (159), but these antibodies are poorly characterized.

b. T cells. Paradoxically, although T cells apparently play the central role in IDDM pathogenesis, few data have been published on T cell reactivity to islet antigens in humans. Of note are pioneering studies using the leukocyte migration assay with islet extracts (160) and, more recently, proliferation assays using human islets, fetal pig islets (161, 162), GAD (152, 153), and hsp 65 (our unpublished observations). The anti-islet T cell response has been best documented in the NOD mouse and the BB rat, where transfer of diabetes can be obtained with purified T cell populations (142, 143, 145), culminating in the production of pathogenic islet-specific T cell clones (144, 146). Successful transfer requires the simultaneous presence of CD4 and CD8 cells when using irradiated recipients that are the most immunoincompetent (142, 163, 164).

4. Immunization and tolerance. Criterion 4 of autoimmune diseases (reproduction of the disease by sensitization against an autoantigen) cannot be met in human diabetes and has very partially been met in animal models, probably due to the uncertain knowledge of the target autoantigen. This is not an absolute criterion even if such a demonstration would greatly aid our understanding of IDDM pathogenesis. The induction in normal animals of insulitis by anti-insulin sensitization (165) and of transient diabetes by immunization against a hsp 65-derived peptide (166) opens the way in this direction. Additionally, two recent studies have shown that insulitis and diabetes can be prevented in NOD mice by injecting them with soluble recombinant GAD at 3 weeks of age either intravenously (167) or intrathymically (168).

5. Indirect evidence. The following indirect evidence exists to support the autoimmune nature of human IDDM: 1) infiltration of the islets of Langerhans by mononuclear cells (insulitis) (169–171); 2) common association of IDDM with other "classical" autoimmune diseases, notably thyroiditis (47); 3) association of IDDM with HLA genes (64–71), which are known to be associated with most autoimmune diseases; and 4) anomalies of the immune system not directly linked to islet cell autoreactivity in human diabetics, such as augmented levels of activated T cells (DR+ and IL-2R+) (172, 173), circulating IL-2 receptor (173, 174), and CD5+ B cells (175). Other abnormalities have been described in animal models, such as lymphocytopenia (14) and increased NK cell activity (176) in BB rats, thymic anomalies in NOD mice (177–181) and BB rats (182), and decreased IL-4 production (183) and delayed T cell apoptosis in NOD mice (80).

VI. β-Cell Target Autoantigens

A. Introduction: the role of β-cell autoantigen(s) in sensitization and lesion formation

The identification of target autoantigens in IDDM is a major challenge for pathogenesis, immunological diagnosis, and immunotherapy. Several candidate autoantigens have been described, but none has so far convincingly been shown to be 'the diabetes autoantigen.' The existence of a precise target autoantigen epitope is suggested by the IDDM association with specific HLA alleles (MHC immune response genes are specific for a given epitope) but one might argue that HLA disease control is not necessarily antigen-specific (MHC genes other than class II genes may explain the HLA-IDDM association). Our recent demonstration that alloxan-treated NOD mice, which lack β-cells, can no longer sustain the survival of pathogenic T cells (184) also supports the hypothesis that the autoimmune response in IDDM is driven by a β-cell autoantigen, as is presumably the case in many if not all organ-specific autoimmune diseases (185). Neonatal thyroidectomy prevents the spontaneous production of antithyroglobulin autoantibodies normally synthesized in the obese chicken (186).

TABLE 3. Immunotherapy of diabetes in NOD mice

Agent	References	Prevention (treatment started ≤3 months of age)	Prevention of diabetes transfer (treatment of the recipient)	Prevention of cytoxan-induced IDDM	Treatment of overt diabetes (treatment started after the onset of hyperglycemia)
Immunomanipulation					
Neonatal thymectomy	303	+			
Allogeneic bone marrow transplantation	226	+			
Backcross to nude mice	9	+			
Backcross to scid mice	10	+			
MHC transgenes	53–58	+			
Intrathymic islet grafting	296	+			
CD4 T cells	246		+		
Immunosuppressive agents					
Cyclosporin	304	+			±/−
FK506	305, 306	+		+	
Deoxyspergualin	307			+	
Rapamycin	308	+			−
ALS	278				+
Monoclonal antibodies					
αCD3	294	+			+
αTCR	262	+		+	+
αVβ8	220			+	
αCD4	118, 293, 309–312	+	+		+
αCD8	313 + (our unpublished data)	+	+	+	+
αclass I	314			+	
αclass II	250	+	+ (neonate)	+	
αIL-2R	315	+			
αCD45RA	178	+			
αγIFN	237, 238		+	+	
α-IL-6	238				
Cytokines					
IL-1	268	+	+		
IL-4	183	+			
TNFα	269, 270	+	+		
IL-2 toxin	316		+		
Miscellaneous					
CFA	120–121, 124a	+			
BCG	124	+		+	
Antioxidants	317	+ (with steroids)			
Aminoguanidine (NO inhibition)	271		+		
Vitamin D_3	318	+ (insulitis)			
Gangliosides	319	+			
Con A	320	+			
hsp65/peptide	16, 17	+			
Insulin (parenteral)	201, 202	+			
Insulin (oral)	200	+			
Diets	88–90	+			± −
Nicotinamide	321	+			
Immunoglobulins	322	+			
Silica	323	+		+	
Peptides	295	+			
Viruses	91–94	+			

+, Suppression of diabetes; −, no effect.

TABLE 4. Immunotherapy of diabetes in BB rats

Agent	References
Immunomanipulation	
Neonatal thymectomy	324
Allogeneic bone marrow transplantation	225
Intrathymic islet grafting	226, 229
Lymphocyte transfusion	254
Immunosuppressive agents	
Cyclosporin	325–327
Anti-lymphocyte sera	328
Anti-class II monoclonal antibodies	329
Anti-IFNγ monoclonal antibodies	239
Miscellaneous	
Total lymphoid irradiation	330
TNFα	331
Low essential fatty acid diet	88
Low protein diet	89, 90
Insulin (parenteral)	203–205

TABLE 5. Immunotherapeutic trials in human IDDM

		Reference
Immunosuppressive agents		
Cyclosporin		147, 148, 297, 300, 301
combination	+ nicotinamide	332
	+ bromocriptine	333
FK506		334
Steroids		335
Azathioprine		299
combination	+ corticoids	298
	+ thymostimulin	336
OKT3		149
IL-2 toxin		149
Miscellaneous		
Nicotinamide		290, 291, 292
Subcutaneous insulin		206
Intravenous immuno-globulins		337
Lymphocyte transfusion		338
Pancreatic irradiation		339
Thymopoietin		340

Alternatively, the anti-islet response could be part of a more global immune hyperreactivity, as in the rat model of generalized autoimmunity obtained after thymectomy and irradiation (23, 24). In this model, pathogenic anti-islet autoimmunity is only the expression of exaggerated physiological autoreactivity due to the loss of immune regulatory function, with no apparent requirement for an autoantigenic driving force.

An intermediate possibility is that β-cell autoantigens do indeed drive the anti-β-cell autoimmune response but that several autoantigens (each with a limited number of dominant epitopes) intervene concomitantly. For unknown reasons (*e.g.* a viral infection), the β-cells might become abnormally immunogenic and stimulate a strong autoimmune response to several of its molecular constituents, provided there is the relevant MHC molecule to present them to T cells. In this hypothesis, either one of these triggering β-cell autoantigens plays a dominant role or MHC genes are not involved in disease susceptibility through conventional Ir genes. There is little room for multiple unrelated autoantigens to share the same precise HLA binding epitopes.

B. Primary and secondary autoimmunization. B and T cell epitopes

It is unlikely that the whole B and T cell response toward a large number of β-cell autoantigens observed in diabetics is primary (or pathogenic). The initial T cell-mediated β-cell lesions probably induce the release of degradation products that in turn elicit the production of secondary B or T cell immune responses. This is suggested by the chronological appearance of T cell proliferative responses to several β-cell autoantigens in the NOD mouse (168). Tolerance induction to the first of these autoantigens prevents onset of reactivity to other autoantigens without reciprocity. This is also probably the case for the anti-islet autoantibodies discussed above. The problem is further complicated in the case of T cells by the fact that these secondary immune responses could contribute to the development of the β-cell lesion and play a significant role in the chronicity of disease. It should be mentioned at this stage that, for obvious reasons of feasibility, most studies aimed at the identification of IDDM autoantigens involve the use of autoantibodies for screening, whereas the initial triggering autoantigen(s) and target autoantigen(s) are recognized by T cells. This is a major pitfall since T and B cell epitopes differ radically: T cell epitopes are sequential whereas B cell epitopes are conformational (187). In addition, T cells can recognize intracytoplasmic proteins that are processed and then exposed at the cell surface in conjunction with MHC molecules, whereas antibodies can only be pathogenic *in vivo* after binding to cell surface molecules.

C. Candidate autoantigens

A number of putative β-cell autoantigens have recently been characterized.

GAD is an enzyme controlling the biosynthesis of the inhibitory neurotransmitter γ-aminobutyric acid. It has recently been identified (151) as one of the 64 kilodalton (kDa) antigens previously detected by immunoprecipitation of islet extracts by diabetics' sera (188). GAD exists in two isoforms of 65 and 67 kDa (189). It is present in β-cells and the brain, and its sequence shows major homology both between the two isoforms and between mammalian species (189, 190). Anti-GAD antibodies were initially found (at high titers) in the stiff-man syndrome, a neurological disease often associated with ICAs and sometimes IDDM (151). They are also found at lower titers (using various techniques: enzyme trapping, immunoenzymatic assays, etc.) in 60–70% of diabetics (191–193) and in most ICA+ prediabetics (194, 195). As mentioned above, T cell proliferation is induced *in vitro* by recombinant GAD preparations in IDDM patients (152, 153), but the antigen specificity of the proliferation remains to be proven with highly purified material. One must formally exclude contamination by highly mitogenic endotoxin of the bacterial recombinant preparation used in these stud-

ies. Although there is little doubt that GAD is one of the major β-cell antigens, the role of this antigen in the pathogenesis of human IDDM remains to be proven. Indeed, anti-GAD antibodies do not appear to be more predictive than ICAs of diabetes onset in prediabetics (194, 195), and a protective role among ICA+ subjects has been indicated by recent studies (196). Conversely, a pathogenic role could be given to GAD-reactive T cells. Recent data obtained in the NOD mouse indicate that administration of GAD in 3-week-old mice, either intravenously (167) or intrathymically (168), prevents the onset of insulitis and diabetes.

A 37-kDa protein is immunoprecipitated by diabetic patients' sera together with a 50-kDa protein instead of the 64-kDa band when islet extracts are treated with trypsin (197). Antibodies directed against the 50-kDa species recognize GAD. They are absorbed by recombinant GAD65, and their presence strictly correlates with that of anti-GAD antibodies. Conversely, antibodies directed against the 37-kDa protein are apparently distinct from anti-GAD antibodies. They are not absorbed by recombinant GAD, suggesting that the 37-kDa protein is derived from a 64-kDa molecule distinct from GAD (198). The anti-37-kDa antibodies seem to be better predictors of diabetes in prediabetics than anti-GAD antibodies (196).

Insulin is a logical candidate for an IDDM autoantigen since it is the best established β-cell-specific differentiation antigen. Its role in the pathogenesis of the disease appears initially unlikely though, since insulin is essentially expressed in the β-cell cytoplasm. However, we have seen that such cytoplasmic antigens can be processed and recognized by T cells. Anti-insulin autoantibodies are often found in prediabetics before treatment with insulin (154). Immunization of normal animals of different species induces insulitis (165), and sensitization of prediabetic NOD mice against insulin can protect them from diabetes when either the parenteral (199) or the oral (200) route is used. This protective effect is presumably linked directly to the immunogenicity of insulin at least when used parenterally, since the functionally inactive insulin B chain can be used instead of native insulin in parenteral sensitization experiments (199). This effect of insulin should be distinguished from the above-mentioned protection conferred by subcutaneous injections of insulin that probably act directly at the β-cell level (201–206).

hsp 65 (65-kDa heat shock protein) (16) and one of its constitutional peptides (17) have been reported to accelerate the onset of diabetes in NOD mice and even to induce *de novo* diabetes in C57BL/6 mice when coupled to a carrier protein (166). The diabetes thus induced is, however, transient and NOD mice are ultimately protected from diabetes. Disease acceleration and protection can be transferred by hsp 65-reactive T cell clones (17), suggesting that the protection could relate to a mechanism of T cell vaccination, in which mice become sensitized against the T cell receptor of hsp 65-reactive T cell clones. hsp 65 Has been found in β-cells and could be a new target autoantigen (T cell epitope). Alternatively, it could act via molecular mimicry.

p69 Protein has been mentioned as a β-cell autoantigen cross-reacting with BSA (128, 129, 134). The anti-p69 response could be stimulated by cow's milk protein administered during the first weeks of life, again via molecular mimicry.

A 38-kDa protein isolated from β-cell insulin secretory granules has been shown to stimulate T cell proliferation in human diabetics' lymphocytes, giving rise to the production of T cell clones (207, 208). This protein could contain important T cell epitopes.

Other candidate antigens include peripherin, a neurone cytoskleton molecule (168, 209), carboxypeptidase H (168, 210), and the ICA-reactive gangliosides (156).

VII. The Loss of Self-Tolerance to β-Cell Antigens

A. Tolerance to self

It is a major feature of the immune system that B and T cells are physiologically tolerant to most self-antigens (*i.e.* there is no pathogenic autoimmune response). This state of T cell self-tolerance is mainly controlled in the thymus, where self-reactive T cell clones that expanded after contact with self MHC molecules present on the thymic epithelium and stroma (positive selection) are eliminated by autoantigen-driven apoptosis (negative selection) (211, 212). This phenomenon does not, however, eliminate all autoreactive clones, particularly those reacting toward subdominant or cryptic epitopes (213) and autoantigens not present in sufficient concentrations in the thymus. These autoreactive clones are controlled by either a phenomenon known as T cell anergy (the autoreactive cells are present but are not activated after binding the antigen) or by the effect of suppressor mechanisms (211–213). There are several examples in transgenic mice where T cells reactive with antigens expressed on β-cells are reactive with the antigens *in vitro* but not *in vivo*. These cells are not truly "anergic" but may be "ignorant" and hence do not engage in an immune response if they are not properly activated (see above the LCMV transgenic mouse model). Finally, the breakdown of self tolerance that characterizes autoimmune diseases can thus occur through three major mechanisms: insufficient intrathymic negative selection, bypass of peripheral anergy, or defective suppression (211–213).

B. T cell repertoire

Most information on islet-reactive T cells in IDDM is derived from the study of NOD mice. This has been facilitated by the production of a number of islet-specific T cell clones, mostly of the CD4 phenotype (144, 146, 214). Some of these clones have been shown to be diabetogenic after transfer into irradiated adult (146) or nonirradiated young NOD recipients (144). The TCR of one of these CD4 clones was recently used as a transgene (215); transgenic mice showed rampant insulitis at a faster rate than the transgene negative NOD littermates but only borderline and inconsistent hyperglycemia.

Encephalitogenic T cell clones obtained after immunization with myelin basic protein use restricted Vα and Vβ TCR

genes (216). It was thus important to search for a possible restriction of Vα and Vβ gene usage of TCR of T cells involved in IDDM pathogenesis. Several approaches have been taken. Phenotypic studies using indirect immunofluorescence with selected anti-Vβ monoclonal antibodies (217) or dot blot hybridization (218) on pancreas sections or extracts are difficult to perform and have yielded no evidence of restriction. The T cell clones mentioned above do not show any clear preference for a given Vα or Vβ (214, 219). The only two studies that have revealed some restriction were based on diabetes prevention by anti-Vβ monoclonal antibodies. An anti-Vβ8 monoclonal was reported to prevent cyclophosphamide-induced diabetes (220) [an observation not reproduced by another team (221)] and an anti-Vβ6 monoclonal inhibited diabetes transfer in irradiated mice (222). It is interesting to note, in this context, that NOD mice backcrossed to other strains to give a strain that congenitally lacks approximately one-half of the conventional TCR Vβ alleles (including Vβ8 but not Vβ6) still develop diabetes (223). Finally, we shall have to await results of studies in progress with anchored polymerase chain reaction at early stages of insulitis to know whether the TCR of T cells infiltrating NOD islets show restricted usage of any particular TCR fragment, at least in the initial stages. This is an important question from both the fundamental and therapeutic viewpoints, since if a restriction exists, one could envisage preventing IDDM by targeting the minor T cell subset expressing the Vβ gene in question. The T cell oligoclonality can also be studied by analyzing TCR junctional sequence variability. Results obtained in our laboratory (manuscript in preparation) indicate that such oligoclonality might exist initially at the islet level but polyclonality rapidly spreads over the pancreas.

C. Location of the anomaly(ies) leading to the pathogenic anti-β-cell autoimmune response

There is no indication in IDDM, as in other organ-specific autoimmune diseases, that the target autoantigen is abnormal. In fact, transplantation studies mentioned above (115–119) showing that destructive insulitis can be transferred to non-diabetes-prone mouse, rat, or human pancreas indicate that the anomaly is located in the immune system. This is corroborated by the observation that reconstitution of (BALB/c × BG)F1 normal mice with stem cells and thymus from NOD mice results in autoimmune insulitis of the (normal) host pancreas (224). Similarly, reciprocal allogeneic bone marrow transplantation between BB rats and a non-autoimmune rat strain shows that the defect leading to diabetes lies in the bone marrow stem cells (225).

All experimental data converge to suggest that the defect is most strongly expressed at the T cell level. The disease is prevented in NOD mice and BB rats by neonatal thymectomy, backcross to athymic animals, and administration of various anti-T cell antibodies (Table 3). Diabetes can be transferred to healthy recipients by purified T cell populations (142, 143, 145) or T cell clones (144, 146). The question then arises as to whether the anomaly is located 1) at the T cell precursor level (in the bone marrow), 2) in the thymus (unable to perform normal negative selection or to differentiate effector or regulatory cells), or 3) at the level of the MHC-autoantigen interaction, which would generate molecular complexes that are highly immunogenic for T cells of diabetes-prone individuals. Evidence has been found in favor of all three hypotheses.

Bone marrow precursor cells contain the "germ" of diabetogenicity, since transplantation of NOD mouse or BB rat bone marrow to nondiabetic strains (after irradiation) leads to diabetes (224, 225), and bone marrow transplantation from human diabetics may lead to rapid diabetes onset in the recipient (116, 117). Conversely, transplantation of 'normal' allogeneic bone marrow prevents diabetes in NOD mice and BB rats (225, 226).

This does not rule out an intrinsic thymus defect, several of which have been identified in the NOD mouse: 1) deficient *in vitro* thymocyte proliferation in response to antigens and mitogens shown recently to be linked to deficient regulation of the $p21^{ras}$ activation pathway (177); and 2), abnormal proportions of CD45RA+ T cells among mature thymocytes (178). These thymocyte abnormalities could relate to the bone marrow defects just discussed. This is less likely the case for abnormal extracellular matrix (with large perivascular spaces filled with lymphocytes) (179, 180), and reticulum (181) and deficient thymic hormone secretion (179). All these anomalies could indicate a defective thymic microenvironment. In the same vein is the decreased expression of class II MHC molecules observed in some areas of the BB rat thymus (182).

The role of MHC molecules has already been discussed. Their central contribution to diabetes susceptibility is clearly established, but it is certainly not sufficient in itself, since the majority of subjects with a predisposing HLA allele never develop the disease. Additionally, one should not equate the HLA-IDDM association to the presence of predisposition immune response genes (HLA-autoantigen peptide presentation), since MHC genes can be involved at several levels not directly related to peptide recognition by T cells.

D. Defective negative selection

This hypothesis is illustrated by the SV40 transgene mouse model mentioned above (31), in which late expression of the T SV40 antigen (after intrathymic negative selection has taken place) leads to anti-T antigen sensitization and insulitis, inasmuch as the T antigen is selectively expressed on β-cells. This mechanism could apply to virus-induced neoantigens. There is little evidence, however, for the existence of such neoantigens either in NOD mice and BB rats or in human IDDM. There is apparently no major abnormality of distribution of T cells expressing the various Vβ fragment expression in NOD mice (218) or BB rats (227), as could have been anticipated if negative selection by a superantigen had created major gaps in the T cell repertoire. In fact, islet-reactive T cells having escaped negative selection are present in normal individuals, as demonstrated by the onset of diabetes in non-autoimmune-prone rats after thymectomy and irradiation (28, 29) and by the induction of diabetes in normal mouse strains after sensitization to hsp 65 peptides (166). In

conclusion, although one cannot exclude it formally, there is little evidence so far in IDDM of a failure for negative selection of β-cell reactive clones. One can assume that physiologically β-cell target autoantigens are not present in the thymus at sufficient concentrations to induce negative selection of the responding T cell clones or that these antigens may be present in the thymus but diabetogenic epitopes are subdominant or cryptic and do not give rise to negative selection. The possibility demonstrated in both the NOD mouse and the BB rat to prevent the onset of IDDM by placing islet grafts (228, 229) or soluble GAD (168) intrathymically is compatible with such a hypothesis.

E. Breakdown of T cell anergy

Anergized T cells are not activated by antigens presented in normal conditions but can differentiate in the presence of large amounts of IL-2. There is no direct evidence of such a mechanism in IDDM, except for the unconfirmed acceleration of diabetes in BB rats after IL-2 administration (230). However, hyperexpression of class I MHC molecules (170, 231–233) and, more controversially, aberrant expression of class II molecules (170, 231, 233–235) could conceivably favor more efficient presentation of β-cell antigens to T cells and thus break down the physiological anergy of islet-reactive T cells (if indeed MHC class II-expressing β-cells can present antigens). The role of IFNγ in MHC class II expression is suggested by the *in vitro* induction of HLA class II molecules in human islet cells by IFNγ (plus TNF) (236) and by the prevention of diabetes by administration of anti-IFNγ monoclonal antibody in NOD mice (237, 238) and in BB rats (239). One must, however, interpret these data with care, even if aberrant expression of class II MHC molecules in β-cells can indeed provoke autoimmune (transferable) insulitis, as shown by the IFNγ transgenic model (32, 33). Alternatively, aberrant expression of class II MHC molecules could be a secondary phenomenon: activated T cells present in the islets produce IFNγ (240) that could induce the aberrant MHC class II molecule expression. Our observation (241) that class II molecule expression appears within a few days after adoptive transfer of diabetogenic spleen cells on pancreatic endothelial cells illustrates this possibility. The absence of abnormal expression of class II MHC molecules reported in prediabetic NOD mice (217, 235) and BB rats (233) and the absence of 'autoimmune' type diabetes in transgenic mice expressing class I (42) or class II (43, 45) MHC molecules in β-cells is compatible with such an alternative hypothesis but in no way proves it. The MHC molecule expression could be too weak in the rodent spontaneous models to be detected by the immunofluorescence technique used in the experiments mentioned (other results reported in Ref. 234) and too high in the transgenic mouse models to provide meaningful information. It should also be mentioned at this stage that T cell-mediated destruction of β-cells can be obtained in the absence of CD4 T cells and MHC class II molecules. Mice that were class II-deficient after a targeted disruption of the $A\beta^b$ gene were bred to transgenic mice expressing the LCMV glycoprotein in β-cells. Such transgenic class II-deficient mice developed diabetes after infection with LCMV (242).

The significance of class I molecule hyperexpression (more consistent in the experimental setting) is complicated by our failure to understand the way in which class I-restricted CD8+ cells contribute to the pathogenesis of IDDM (see below).

Another interesting mechanism is based on the phenomenon of molecular mimicry already mentioned for GAD [cross-reactivity with a Coxsackie B viral protein (114)] and p69 (cross-reactivity with BSA) (128, 129). In this mechanism, the extrinsic antigen to which T cells are not tolerant serves as a carrier for the tolerized cross-reactive T cell epitopes of the autoantigen, leading to a bypass of self-tolerance. It will be important to characterize further the cross-reactive epitopes. Some data have been reported for BSA and p69: the ABBOS peptide is a 17-amino acid residue long peptide shared between BSA and the β-cell p 69 antigen (129). The case of GAD is less well documented since the sequence homology is modest (114). If this molecular mimicry holds true, it would remain to be learned how the chronic autoimmune T cell response is maintained after the disappearance of the cross-reactive external antigen. In the well documented case of rheumatic fever the autoimmune reaction ceases when the antigen (streptococcus) disappears. Perhaps one could think that the initial anti-β cell immune response triggers an anti-idiotype response that would perpetuate the anti-β-cell response within idiotype networks the initial response or more simply that the initial lesion inducing spread sensitization against other β-cell autoantigens released by the first aggression.

F. Defective suppression

The existence and function of suppressor T cells have been the subject of a heated debate among immunologists over the last 10 yr. A number of experimental data suggest that a defect of these suppressor cells might contribute to the onset of diabetes in rodent models of diabetes (243).

In the NOD mouse, diabetes onset is accelerated by thymectomy performed at 3 weeks of age (244) and by administration of cyclophosphamide (11, 12), a drug known for its selective effects on suppressor T cells. Diabetes transfer is only obtained in immunodeficient recipients, *i.e.* neonates (142) and adults that have been sublethally irradiated (144) or thymectomized and treated with an anti-CD4 monoclonal antibody (245). One can prevent diabetes transfer by spleen cells from diabetic mice by preinfusion of CD4 spleen cells from nondiabetic syngeneic mice (246). CD4 and CD8 suppressor clones have been reported (247–249), as has the production of a suppressor factor (249). Treatment of young NOD mice with an anti-class II monoclonal antibody protects them from diabetes, and this protection is transferable to non-antibody-treated mice by infusion of CD4 T cells from protected mice (250). Similarly, staphylococcal superantigens (SEA and SEB) prevent the onset of diabetes in NOD mice (251), and this protection is also conferable to naive NOD mice by transfer of CD4 T cells from superantigen-treated mice. Also of interest here is the intriguing observation that

diabetes can be prevented in NOD mice by injection of autologous spleen cells exposed *in vitro* to cyclosporin and IL-2 (252).

In the BB rat the disease is accelerated by the administration of an anti-RT6 monoclonal antibody (253) and prevented by transfusion of lymphoid cells from diabetes-resistant DR BB rats (254).

The mechanisms of this defective suppression are still unknown but could involve an abnormal shift of TH2 cells toward TH1 cells of the islet-reactive CD4 T cells. It has been shown that CD4 T cells comprise two subsets—TH1 cells (that produce IL-2 and IFNγ) and TH2 cells (that produce IL-4 and IL-10)—that oppose each other by reciprocal down-regulation. TH1 cells are essentially involved in cell-mediated immune responses, whereas TH-2 cells are involved in helping antibody-forming cells (255). The abnormal shift from TH2 to TH1 islet-reactive T cells is supported by the low IFNγ/IL-4 ratio found in noninvasive insulitis, contrasting with a high ratio in invasive insulitis (240), and by the recent observation that IL-4 (whose production is deficient in the NOD mouse thymus) reverses the T cell proliferative unresponsiveness in NOD thymocytes and delays the onset of diabetes in NOD mice (183). This hypothesis is also in keeping with the inverse relationship between humoral and cellular immunity to GAD in subjects at risk for IDDM (194).

The following findings also support the role of suppressor mechanisms: I-A^k transgenic mice that are protected from diabetes (53) become diabetic after cyclophosphamide treatment, and their spleen cells can transfer diabetes in immunodeficient hosts (256); similarly, spleen cells from I-A^d transgenic NOD mice that are protected from diabetes prevent the diabetogenic capacity of splenocytes from overtly diabetic NOD mice (257). Introduction of I-E in transgenic NOD mice also protects from diabetes (56, 57) through a mechanism that could involve suppressor cells.

G. Conclusions

It is difficult to formulate a global hypothesis explaining the loss of self-tolerance to islet antigens in diabetic subjects. The disease is heterogeneous and multifactorial: several mechanisms may simultaneously be at work, superimposed on a particularly efficacious MHC-controlled recognition of β-cell autoantigen peptides. An attractive hypothesis is a particularly immunogenic expression at the β-cell surface of a subdominant or cryptic autoantigen not having induced intrathymic negative selection. This abnormal expression could be secondary to a viral infection known to modify HLA gene expression through IFN production, but many other cellular events could play a similar role, including endogenous β-cell genetically controlled peculiarities. In this case, as mentioned above, it might be that more than one antigen molecule or epitope shows increased immunogenicity, providing an explanation for the diversified anti-β-cell B and T cell immune response. Alternatively, one epitope could initiate the autoimmune responses [*e.g.* GAD as suggested by the chronology of appearance of the islet T cell reactivity and by spread tolerance after GAD administration (167, 168)].

Another hypothesis involves the expression of a neoantigen at the β-cell surface secondary to the effect of a viral infection or a chemical. Finally, one may think of a bypass of anergized T cells by molecular mimicry after stimulation by an environmental factor (such as a virus or a cow's milk protein).

In all these hypotheses, an important role should be given to defective suppressor mechanisms amplifying the autoimmune response. Primary deficiency of regulatory T cells may give rise to autoimmune reaction as in the models of post thymectomy (and irradiated) models of autoimmunity. However, in view of the usually β-cell-restricted autoimmunity observed in human diabetes, it is unlikely that suppressor cell deficiency can by itself represent a sufficient factor to induce IDDM in most cases.

VIII. The β-Cell Lesion

A. Insulitis

The islets of Langerhans of recently diagnosed diabetic patients are infiltrated by mononuclear cells (insulitis) (169). These mononuclear cells include a majority of T cells (belonging to the two major subtypes CD4 and CD8, with apparently a predominance of CD8+ cells) and macrophages (170, 171). Some B cells may also be present. Fewer than 10% of β-cells persist 2–4 months after initiation of insulin therapy, as recently demonstrated by a pancreas biopsy study (258). This atrophy is selective for β-cells since other endocrine cells remain intact.

Studies of rodent models (217, 241, 259) have shown that destructive and invasive insulitis is preceded by periinsulitis (mononuclear cell infiltrate around the islets) and peripheral insulitis (lymphocytes at the islet periphery). Infiltrating T cells again include both CD4 and CD8 T cells, with signs of activation (IL-2 receptor expression). Transfer studies (217, 241) have shown that CD4 cells are the first cells to invade the islets. Interestingly, in the absence of CD4 cells (transfer of purified CD8 cells), CD8 cells do not migrate to the islets (241). Adhesion molecules (L-selectin) and very late antigen 4 (VLA 4) receptors may be involved in mediating leukocyte homing to the islets since insulitis and diabetes are inhibited in NOD mice by blocking these molecules by specific monoclonal antibodies (260). Immunohistological studies have shown that the infiltrating T cells express various cytokines such as IFNγ and IL-4, with a tendency for low IFNγ production and high IL-4 production in noninvasive insulitis contrasting with high IFNγ and low IL-4 levels in invasive insulitis (240), an interesting pattern which still requires confirmation. Importantly, there is no significant immunoglobulin deposition.

Studies of pancreatic sections in diabetic patients have revealed hyperexpression of class I and aberrant expression of class II MHC molecules at disease onset (170, 231). This important observation has been the subject of controversial findings in rodent models (232–235).

B. Inflammation vs. atrophy

It is important to know whether the β-cell dysfunction characteristic of IDDM is only due to β-cell destruction (atrophy) or can involve, in the early stages of clinical diabetes, a reversible functional inhibition (inflammation) leaving room for immunointervention at advanced stages of the disease. The latter is strongly supported by two sets of observations made in NOD mice. First, islets from recently diabetic NOD mice, which initially show low insulin production, regain part of their function when cultured *in vitro* in the absence of autologous T cells (261). Second, a single injection of an anti-TCR monoclonal antibody in NOD mice with established diabetes induces rapid normalization of glycemia (lasting throughout treatment) (262). This functional recovery must be distinguished from that observed in recently diagnosed diabetes after the start of intensive insulin therapy (263). The observed increase in C peptide production is then due to the release from glucotoxicity afforded by insulin.

C. Unique β-cell fragility

β-Cells appear to be particularly fragile cells, sensitive to a wide array of aggression. As mentioned above, hyperglycemia tends to reduce insulin secretion in addition to inducing peripheral insulin resistance. It is not known whether the relief from glucotoxicity explains the β-cell protection afforded by insulin therapy in NOD mice (201, 202), BB rats (203–205), and human prediabetics (206). It has been proposed that insulin could act by reducing the expression of β-cell autoantigens, but insulin may also prevent transient episodes of deleterious hyperglycemia.

Various cytokines can alter β-cells or even destroy them. This is particularly the case for IL-1 (264) and TNF (265), which are most active in combination. The effect of IL-1 is not totally β-cell-specific though, since α-cells are also affected and low IL-1 concentrations are only deleterious at supraphysiological glucose levels (266). In addition, administration of recombinant IL-1 induces hypoglycemia rather than hyperglycemia in normal and diabetic db/db and ob/ob mice (124) and prevents diabetes in NOD mice (267, 268). Similarly, TNFα, which shares many *in vitro* properties with IL-1, induces protection rather than acceleration of diabetes in NOD mice (269, 270) and BB rats (Tables 3 and 4). Other mediators could also intervene, possibly under cytokine control, such as nitrite oxide (NO), whose product is increased in NOD mouse islets (271) and whose inhibition by aminoguanidine delays the onset of diabetes in a transfer model (271).

It is not known whether β-cells from IDDM patients intrinsically show abnormally high fragility compared to those from healthy subjects. Pancreas and islet transplantation experiments mentioned above do not argue in this direction, since β-cells from non-diabetes-prone individuals appear to be fully sensitive to the effector mechanisms responsible for diabetes, as shown in NOD mice (118), BB rats (119), and humans (115–117).

D. Conclusions: the nature of pathogenic effector mechanisms (cell-mediated cytotoxicity or lymphokine effect ?)

Anti-islet cell autoantibodies are produced in large amounts in both rodent and human IDDM. There is no evidence, however, that these autoantibodies are pathogenic, even in the case of those directed at β-cell surface determinants. As just mentioned, no immunoglobulin deposits are found in islets. The disease cannot be transferred by serum of affected mice, whereas diabetes can be transferred by purified T cells in NOD recipients, even when the latter have been rendered unable to synthesize antibodies by perinatal anti-immunoglobulin M monoclonal antibody treatment (272).

T cells are beyond any doubt the main β-cell aggressors. Diabetes can be transferred to nondiabetic syngenic animals by purified T cells from diabetic NOD mice (142, 145) or BB rats (143) or T cell clones (144, 146) derived from diabetic NOD mice. Furthermore, selective T cell elimination by an anti-TCR monoclonal antibody normalizes hyperglycemia in diabetic NOD mice, as previously mentioned (262).

In contrast, there is still great uncertainty as to the intimate mechanisms of T cell-mediated aggression toward β-cells. Direct antigen-specific CD8+ T cell-mediated cytotoxicity is a logical hypothesis, since CD8 T cells are predominant in human IDDM-associated insulitis (170, 171). Additionally, CD8+ T cells are necessary to transfer diabetes to fully immunoincompetent irradiated or neonatal NOD mice (9, 10, 142, 146, 163) and BB rats (164). Also, NOD mice backcrossed with CD8 T cell-deprived mice whose MHC class I genes have been inactivated by homologous recombination do not develop diabetes (273). There is some evidence that CD8 T cells from diabetic patients and animals lyse β-cells (146, 274) but these results have been difficult to reproduce. CD8 T cells expressing the cytolytic mediator perforin are found in NOD mouse insulitis (275), but this mediator is found in most cytotoxic cells and not exclusively in antigen-specific cytolytic T lymphocytes. CD8 T cells have also been shown to inhibit insulin release by islet cells cultured *in vitro* (276), but the interpretation is complicated by the absence of MHC restriction in this model.

Diabetes can be transferred to young NOD mice by CD4 T cell clones alone (144, 146), even after administration of an anti-CD8 monoclonal antibody to rule out any involvement of host CD8 T cells (10, 277). This observation is at variance with previously mentioned evidence that CD8+ T cells are necessary for diabetes transfer. Perhaps young NOD mice (3–4 weeks) used for T cell clone transfer have some CD8+ T cells (even after anti-CD8 antibody treatment) that cooperate with the CD4 T cell clones. CD8 T cell clones have not proven capable of transferring the disease (146) but the addition of polyclonal CD8+ T cells from diabetic mice accelerates diabetes transfer by CD4+ T cell clones in irradiated recipients (146).

T cells could also intervene by secreting various lymphokines that can be directly toxic to β-cells or attract in the pancreas and activate other cell types such as monocytes, macrophages, and eosinophils all found in insulitis. These cells could in turn produce β-cell-toxic mediators such as IL-

1 or TNF to which, as mentioned above, β-cells are exquisitely sensitive. The prevention of diabetes obtained in rodent models by treatment with antioxidants, desferrioxamine, or nicotinamide (Table 3) fits with this hypothesis, suggesting the pathogenic role of free radicals and, possibly, nitric oxide.

Such a role of lymphokines, known to be primarily produced by CD4+ T cells (rather than CD8+ T cells), is supported by the already mentioned capacity of CD4+ T cell clones to transfer diabetes (144, 146, 277) and the recurrence of diabetes after transplantation of MHC-incompatible islet grafts in NOD mice (118) or BB rats (119) in conditions excluding allogeneic rejection (prior islet culture *in vitro*): cytotoxic T lymphocytes cannot exert their activity against an MHC-incompatible target because of the MHC restriction of antigen recognition by T cells. It is also interesting to note that anti-CD4 monoclonal antibodies prevent recurrence of diabetes in islets grafted in NOD mice, whereas anti-CD8 monoclonals do not (118); however, this must be interpreted with caution since, in another model, both anti-CD4 and anti-CD8 monoclonals prevent cyclophosphamide-induced diabetes (our unpublished observation). The interpretation of these contradictory data should perhaps take into account the fact that when administered several days before grafting (as performed in the experiments just mentioned) (118), anti-CD4 monoclonals can induce long-term anti-islet unresponsiveness, which anti-CD8 monoclonals cannot (278).

Finally, the question of the respective involvement of CD4+ T cell-produced lymphokines and of CD8+ T cell-mediated cytotoxicity remains open, since none of the arguments supporting the role of one or the other provides absolute proof. The problem is complicated by the helper function of CD4+ T cells for CD8+ T cell differentiation. Alternatively, IFNγ produced by CD8+ T cells could enhance CD4+ T cell action. In conclusion, one may reasonably assume from data presented above that both subsets are needed for diabetes since elimination of either subset can prevent diabetes in NOD mice and BB rats. It is still difficult to say which cell exerts the central effector function and how each cell type regulates the other.

Attention should also be given in this context to the possible cytotoxic activity of natural killer (NK) cells and lymphokine-activated killer (LAK) cells that exert antigen-nonspecific cytotoxicity activated by lymphokines. There is some evidence in BB rats that such cells could play a significant role (176).

IX. Clinical Implications

The data and concepts discussed above have already generated a number of clinical applications and hold exciting prospects.

A. New appreciation of disease heterogeneity

When genetic factors and immune mechanisms are better defined, a new classification of diabetes mellitus will undoubtedly be formulated, distinguishing autoimmune diabetes from nonautoimmune diabetes.

Autoimmune diabetes will cover most (but not necessarily all) patients currently listed as having type 1 diabetes. It will also include the large number of patients with NIDDM due to a slow autoimmune anti-β-cell reaction. These patients are recognized by the presence of ICAs and the predisposing alleles DR3 and/or DR4. The proportion of slow type 1 among NIDDM patients reaches 10–15% according to studies (279–283). Identification of these patients, for example by ICA screening of NIDDM patients, is clinically important because of the possibility of early insulin therapy, which eventually becomes necessary in most of these patients after a long period of poor metabolic control (279, 281, 282).

A special place should be reserved for diabetes due to the direct cytolytic effect of viruses on β-cells [*e.g.* rubella (101)] and toxic agents [*e.g.* pentamidine (127)], even if the involvement of the immune system cannot be ruled out in these cases.

Finally, attention must be paid to nonDR3-nonDR4 fully insulin-dependent diabetics. The level of ICAs and sensitivity to cyclosporine are lower in these patients (67), who could represent an interesting etiological subgroup.

In fact, the question must be raised of the extent of IDDM heterogeneity. One may be lured by the study of the NOD mouse and the BB rats which, as mentioned above, represent only a single individual produced in multiple copies. The etiological role of multiple factors (genetic and environmental) is firmly established, but it is difficult to say whether all these factors intervene in a single patient or whether a limited number of them is involved in various combinations in individual subjects explaining the disease heterogeneity.

B. Predicting diabetes

We have seen that ICAs (and other islet-reactive autoantibodies) can be detected several years before the clinical onset of diabetes (284). These immunological markers, combined with the identification of predisposition genes (HLA and non-HLA genes), allow a fairly precise prediction of the disease risk in families of diabetic patients [~80% at 5 yr (see reviews in Refs. 285–289)]. ICAs and anti-insulin autoantibodies appear to be the best predictive markers at present. Anti-GAD and β-cell-restricted ICAs (which essentially include anti-GAD antibodies) appear to show a weaker association with diabetes onset and could even be a marker of protection (196, 289). One must realize, however, that genetic prediction will never exceed the concordance rate in identical twins (35–40%) and that HLA typing will never exceed the concordance rate in HLA-identical siblings (10–15%). Autoantibodies (whatever the test used) are absent in 15–20% of patients with recent-onset diabetes. Perhaps this gap could be filled by T cell assays, but none are yet operational. The complementary use of metabolic tests [assays of precocious insulin secretion following glucose infusion (284)] has not proven very informative, because of the high variability of the response in normal subjects and the late occurrence of interpretable anomalies (only a few months before the onset of insulin dependency). The size of most families in Western countries being small, it is less likely that a prediabetic subject will have a diabetic sibling, and genetic and immunological tests are less efficient in the general population than within

affected families. All these limitations call for renewed research efforts to provide reliable prediction to the degree required for immunotherapy.

C. Immunotherapy

Immunotherapy can be used in human IDDM at three different stages of the disease.

Prediabetes without insulin requirement or even metabolic abnormalities after glucose infusion (to be distinguished from subjects who have all predisposing genes but in whom there is no evidence whatsoever of the initiation of the anti-β-cell autoimmune response). This is the ideal situation since a large fraction of the β-cell mass is still likely to be present and there are strong indications that the autoimmune response is more sensitive to immunointervention at this stage than later on. Unfortunately, only insulin prophylaxis has so far had any activity at this stage (206). Nicotinamide is being tested on the basis of suggestive nonrandomized preliminary studies (290–292).

Preclinical diabetes, where metabolic abnormalities are sufficiently marked to be detected by provocation tests but not to induce an insulin requirement. Slow type 1 diabetes can be placed in this category.

Overt diabetes, defined by insulin dependence. Immunointervention may still be efficacious at this stage, inasmuch as it is started within 6–8 weeks after the initiation of insulin therapy. It should be realized, however, that only a few β-cells are left at this time and one cannot expect a complete and long-term recovery of β-cell function at this stage. In this case, the objective is limited to preservation of the remaining β-cell mass (with possible improvement of β-cell function due to the action on local immunologically mediated inflammation). Even in cases where insulin cannot be withdrawn, a significant improvement of metabolic control may result due to the better efficacy of endogenously produced insulin in response to glucose stimulations than that of fixed insulin injections.

The large array of methods and products that have been successfully used in animal models have already been discussed (Tables 2 and 3). It must be stressed, however, that most of these interventions were applied early in the natural history of the disease, at a phase of "prediabetes" that is difficult to detect reliably in man. In addition, there are ethical problems involved in chronic treatment of young, apparently healthy, subjects. Hence the interest in products active on established diabetes (cyclosporin, monoclonal antibodies) and even more in products inducing long-term unresponsiveness (tolerance) without the need for continuous treatment. This objective has recently proven feasible in NOD mice, with polyclonal antilymphocyte sera (278), and both anti-CD4 (278, 293) and anti-CD3 (294) monoclonal antibodies. The mechanism of the tolerance induction in these experiments is not known but could involve stimulation of regulatory cells (TH2?) by T cells *in situ* under the cover of the anti-T cell antibody. Alternative experimental approaches to antigen-specific immunotherapy include peptide therapy (autoantigen peptide analogs binding to MHC molecules) (295) and intrathymic islet grafting, in an attempt to induce negative selection of islet-reactive T cells (228, 229, 296). One should also mention the attempt to induce specific unresponsiveness (tolerance) in young (3- to 6-week-old) NOD mice using insulin given orally (200) or GAD given either intravenously (167) or intrathymically (168). It is interesting that in the oral insulin model the hypothesis has been put forward that tolerance to the introduced autoantigen leads to spread tolerance toward other β-cell autoantigens, possibly by local production of immunosuppressive cytokines such as TGFβ.

Therapeutic trials in human IDDM have as yet been limited to a small number of compounds, essentially in recent-onset diabetes. Two drugs have proven efficacious in randomized studies: cyclosporin (*vs.* a placebo) (147, 148, 297) and azathioprine in association with steroids (298). One trial using low dose azathioprine (2 mg/kg/day) alone did not show any effect (299). In any case, the remission induced by these two agents was not indefinite (1–3 yr) (300) due to the occurrence of insulin resistance (301, 302) and to the autonomous nonimmunologically mediated deterioration of the remaining β-cell population induced by persistent hyperglycemia (glucotoxicity). However, one cannot exclude the persistence in these patients of an ongoing anti-β-cell autoimmune response since insulin resistance and glucotoxicity are not sufficient in the majority of type 2 diabetics to induce progressive β-cell destruction. Also, the rate of remission was no higher than 50%, and immunosuppressive therapy could not be stopped without rapid relapse.

Based on animal model data, three directions are being taken to circumvent these difficulties: 1) earlier therapy, based on prediction tests and using nontoxic drugs; 2) more acute intervention to improve efficacy and rapidity of action over the relatively slow-acting conventional immunosuppressive drugs [*e.g.* with IL-2/toxin conjugates (149)]; and 3) tolerance induction with either (oral or intravenous) autoantigen administration or monoclonal antibodies.

These approaches are being complemented by better usage of optimized insulin therapy and strict selection of patients for clinical trials.

X. Conclusions and Summary

IDDM is unquestionably an autoimmune disease, as reflected by the presence of β-cell-reactive autoantibodies and T cells, T cell-mediated transfer of the disease in nondiabetic mice, rats, and humans, and disease sensitivity to immunosuppressive therapy. T cells are predominantly, if not exclusively, involved in creating the islet lesions that lead to β-cell atrophy after a stage of reversible inflammation. A full understanding of the disease pathogenesis will require a better definition of the nature of the triggering and target autoantigen(s) and of the effector mechanisms (cytokines, cytotoxic cells?).

Much less information is available on the etiology than on the pathogenesis. Genetic factors are mandatory and the involvement of predisposition genes (HLA and non-HLA) is now being unravelled. The modulatory role of environmental factors is demonstrated by the high disease discordance rate in identical twins and by experimental data showing positive

and negative modulation of the disease by a number of agents, notably infectious agents and food constituents. It is not clear, however, whether a given environmental factor, *e.g.* a precise virus or a cow's milk component, plays a real etiological role in a selected genetic background. IDDM thus appears as a multifactorial disease. It is not known, however, whether all factors intervene concomitantly in a given individual or separately in subsets of patients, explaining the clinical heterogeneity of the disease.

The mechanisms underlying the loss of tolerance to self β-cell autoantigen(s) are still unknown. Defective intrathymic negative selection of autoantigen-specific autoreactive T cell clones is unlikely. Breakdown of T cell anergy could occur according to various mechanisms, including aberrant expression of MHC molecules and molecular mimicry. Defective suppressor T cell function, perhaps related to TH1/TH2 imbalance, probably intervenes by amplifying the anti-β-cell autoimmune response whatever its triggering mechanism.

Before putative etiological agents are identified, one must base immunotherapy on nonantigen-specific agents. Results recently obtained in NOD mice indicate that the goal of nontoxic long-lasting immune protection from the disease is feasible if treatment is started early enough. In some cases (anti-T cell monoclonal antibodies), it appears that specific unresponsiveness can be induced. This double strategy (early intervention, tolerance induction) is the main challenge for immunodiabetologists. They must convince clinical diabetologists, the patients, and their families that immunoprevention of the disease will only be achievable if research on prediction and immunotherapy proceeds hand in hand. Prediction programs are difficult to run without proposing a safe and potentially efficacious preventive therapy, and the search for therapy cannot be successful without access to prediabetics or patients with preclinical diabetes, who can only be identified in prediction clinics. Hopefully this review will contribute in a modest way to generating the necessary faith in the future of immunoprevention of the disease, which could eventually lead to its eradication.

References

1. Rossini AA, Greiner DL, Friedman HP, Mordes JP 1993 Immunopathogenesis of diabetes mellitus. Diabetes Rev 1:43–75
2. Honeyman MC, Harrison LC 1993 The immunologic insult in type 1 diabetes. Springer Semin Immunopathol 14:253–274
3. Castano L, Eisenbarth GS 1990 Type-1 diabetes: a chronic autoimmune disease of human, mouse, and rat. Annu Rev Immunol 8:647–679
4. Bach JF 1988 Mechanisms of autoimmunity in insulin-dependent diabetes mellitus. Clin Exp Immunol 72:1–8
5. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y 1980 Breeding of a non-obese, diabetic strain of mice. Exp Anim 29:1–13
6. Pozzilli P, Signore A, Williams AJ, Beales PE 1993 NOD mouse colonies around the world: recent facts and figures. Immunol Today 14:193–196
7. Bernard NF, Ertug F, Margolese H 1992 High incidence of thyroiditis and anti-thyroid autoantibodies in NOD mice. Diabetes 41:140–46
8. Baxter AG, Mandel TE 1991 Hemolytic anemia in non-obese diabetic mice. Eur J Immunol 21:2051–2055
9. Matsumoto M, Yagi H, Kunimoto K, Kawaguchi J, Makino S, Harada M 1993 Transfer of autoimmune diabetes from diabetic NOD mice to NOD athymic nude mice: the roles of T cell subsets in the pathogenesis. Cell Immunol 148:189–197
10. Christianson SW, Shultz LD, Leiter EH 1993 Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4+ and CD8+ T-cells from diabetic *versus* prediabetic NOD.NON-Thy-1a donors. Diabetes 42:44–55
11. Harada M, Makino S 1984 Promotion of spontaneous diabetes in non-obese diabetes-prone mice by cyclophosphamide. Diabetologia 27:604–606
12. Yasunami R, Bach JF 1988 Anti-suppressor effect of cyclophosphamide on the development of spontaneous diabetes in NOD mice. Eur J Immunol 18:481–484
13. Nakhooda AF, Like AA, Chappel CI, Murray FT, Marliss EB 1977 The spontaneously diabetic Wistar rat. Metabolic and morphologic studies. Diabetes 26:100–112
14. Poussier P, Nakhooda AF, Faik JA, Lee C, Marliss EB 1982 Lymphopenia and abnormal lymphocyte subsets in the "BB" rat: relationship to the diabetic syndrome. Endocrinology 110:1825–1827
15. Greiner DL, Handler ES, Nakano K, Mordes JP, Rossini AA 1986 Absence of the RT-6 T cell subset in diabetes-prone BB/W rats. J Immunol 136:148–151
16. Elias D, Markovits D, Reshef T, Van Der Zee R, Cohen IR 1990 Induction and therapy of autoimmune diabetes in the non-obese diabetic (NOD/Lt) mouse by a 65-kDa heat shock protein. Proc Natl Acad Sci USA 87:1576–1580
17. Elias D, Reshef T, Birk OS, Van Der Zee R, Walker MD, Cohen IR 1991 Vaccination against autoimmune mouse diabetes with a T-cell epitope of the human 65-kDa heat shock protein. Proc Natl Acad Sci USA 88:3088–3091
18. Like AA, Rossini AA 1976 Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. Science 193:415–417
19. Malaisse WJ 1982 Alloxan toxicity to the pancreatic B-cell. A new hypothesis. Biochem Pharmacol 31:3527–3534
20. Kolb H 1987 Mouse models of insulin dependent diabetes: low-dose streptozocin-induced diabetes and nonobese diabetic (NOD) mice. Diabetes Metab Rev 3:751–778
21. Paik SG, Fleischer N, Shin SI 1980 Insulin-dependent diabetes mellitus induced by subdiabetogenic doses of streptozotocin: obligatory role of cell-mediated autoimmune processes. Proc Natl Acad Sci USA 77:6129–6133
21a. Gerling IC, Friedman H, Greiner DL, Shultz LD, Leiter EH 1994 Multiple low-dose streptozocin-induced diabetes in NOD-scid/scid mice in the absence of functional lymphocytes. Diabetes 43:433–440
22. Weide LG, Lacy PE 1991 Low-dose streptozotocin-induced autoimmune diabetes in islet transplantation model. Diabetes 40:1157–1162
23. Cockfield SM, Ramassar V, Urmson J, Halloran PF 1989 Multiple low dose streptozotocin induces systemic MHC expression in mice by triggering T cells to release IFN-gamma. J Immunol 142:1120–1128
24. Campbell IL, Oxbrow L, Koulmanda M, Harrison LC 1988 IFN-gamma induces islet cell MHC antigens and enhances autoimmune, streptozotocin-induced diabetes in the mouse. J Immunol 140:1111–1116
25. Orlow S, Yasunami R, Boitard C, Bach JF 1987 Induction précoce du diabète chez la souris NOD par la streptozotocine. C R Acad Sci [III] 304:77–78
26. Ihm SH, Lee KU, McArthur RG, Yoon JW 1990 Predisposing effect of anti-beta cell autoimmune process in NOD mice on the induction of diabetes by environmental insults. Diabetologia 33:709–712
27. Taguchi O, Takahashi T, Nishizuka Y 1990 Self-tolerance and localized autoimmunity. Curr Opin Immunol 2:576–581
28. Fowell D, Mason D 1993 Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4+ T cell subset that inhibits this autoimmune potential. J Exp Med 177:627–636
29. Stumbles PA, Penhale WJ 1993 IDDM in rats induced by thymectomy and irradiation. Diabetes 42:571–578
30. McKeever U, Mordes JP, Greiner DL, Appel MC, Rozing J, Handler ES, Rossini AA 1990 Adoptive transfer of autoimmune diabetes and thyroiditis to athymic rats. Proc Natl Acad Sci USA 87:7618–7622

31. Adams TE, Alpert S, Hanahan D 1987 Non-tolerance and autoantibodies to a transgenic self antigen expressed in pancreatic beta cells. Nature 325:223–228
32. Sarvetnick N, Liggitt D, Pitts SL, Hansen SE, Stewart TA 1988 Insulin-dependent diabetes mellitus induced in transgenic mice by ectopic expression of class II MHC and interferon-gamma. Cell 52:773–782
33. Sarvetnick N, Shizuru J, Liggitt D, Martin L, McIntyre B, Gregory A, Parslow T, Stewart T 1990 Loss of pancreatic islet tolerance induced by beta-cell expression of interferon-gamma. Nature 346:844–847
34. Stewart TA, Hultgren B, Huang X, Pitts-Meek S, Hully J, MacLachlan NJ 1993 Induction of type I diabetes by interferon-alpha in transgenic mice. Science 260:1942–1946
35. Higuchi Y, Herrera P, Muniesa P, Huarte J, Belin D, Ohashi P, Aichele P, Orci L, Vassalli JD, Vassalli P 1992 Expression of a tumor necrosis factor alpha transgene in murine pancreatic beta cells results in severe and permanent insulitis without evolution towards diabetes. J Exp Med 176:1719–1731
36. Picarella DE, Kratz A, Li CB, Ruddle NH, Flavell RA 1993 Transgenic tumor necrosis factor (TNF)-alpha production in pancreatic islets leads to insulitis, not diabetes. Distinct patterns of inflammation in TNF-alpha and TNF-beta transgenic mice. J Immunol 150:4136–4150
37. Wogensen L, Huang X, Sarvetnick N 1993 Leukocyte extravasation into the pancreatic tissue in transgenic mice expressing interleukin 10 in the islets of Langerhans. J Exp Med 178:175–185
38. Oldstone MB, Nerenberg M, Southern P, Price J, Lewicki H 1991 Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. Cell 65:319–331
39. Roman LM, Simons LF, Hammer RE, Sambrook JF, Gething MJ 1990 The expression of influenza virus hemagglutinin in the pancreatic beta cells of transgenic mice results in autoimmune diabetes. Cell 61:383–396
40. Lo D, Freedman J, Hesse S, Palmiter RD, Brinster RL, Sherman LA 1992 Peripheral tolerance to an islet cell-specific hemagglutinin transgene affects both CD4+ and CD8+ T cells. Eur J Immunol 22:1013–1022
40a. Scott B, Liblau R, Degermann S, Marconi LA, Ogata L, Caton AJ, McDevitt HO, Lo D 1994 A role for non-MHC genetic polymorphism in susceptibility to spontaneous autoimmunity. Immunity 1:73–82
41. Ohashi PS, Oehen S, Buerki K, Pircher H, Ohashi CT, Odermatt B, Malissen B, Zinkernagel RM, Hengartner H 1991 Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. Cell 65:305–317
42. Allison J, Campbell IL, Morahan G, Mandel TE, Harrison LC, Miller JF 1988 Diabetes in transgenic mice resulting from overexpression of class I histocompatibility molecules in pancreatic beta cells. Nature 333:529–533
43. Markmann J, Lo D, Naji A, Palmiter RD, Brinster RL, Heber-Katz E 1988 Antigen presenting function of class II MHC expressing pancreatic beta cells. Nature 336:476–479
44. Gotz J, Eibel H, Kohler G 1990 Non-tolerance and differential susceptibility to diabetes in transgenic mice expressing major histocompatibility class II genes on pancreatic beta cells. Eur J Immunol 20:1677–1683
45. Bohme J, Haskins K, Stecha P, Van Ewijk W, Lemeur M, Gerlinger P, Benoist C, Mathis D 1989 Transgenic mice with I-A on islet cells are normoglycemic but immunologically intolerant. Science 244:1179–1183
46. Epstein PN, Overbeek PA, Means AR 1989 Calmodulin-induced early-onset diabetes in transgenic mice. Cell 58:1067–1073
47. Rotter JI, Vadheim CM, Rimoin DL 1990 Genetics of diabetes mellitus. In Rifkin H, Porte D (eds) Diabetes Mellitus. Theory and Practice, ed 4. Elsevier, Amsterdam, pp 378–413
48. Lo SS, Tun RY, Hawa M, Leslie RD 1991 Studies of diabetic twins. Diabetes Metab Rev 7:223–238
49. Singal DP, Blajchman MA 1973 Histocompatibility (HL-A) antigens, lymphocytotoxic antibodies and tissue antibodies in patients with diabetes mellitus. Diabetes 22:429–432
50. Platz P, Jakobsen BK, Morling N, Ryder LP, Svejgaard A, Thomsen M, Christy M, Kromann H, Bent J, Nerup J, Green A, Hauge M 1981 HLA-D and -DR antigens in genetic analysis of insulin dependent diabetes mellitus. Diabetologia 21:108–115
51. Todd JA, Aitman TJ, Cornall RJ, Ghosh S, Hall JR, Hearne CM, Knight AM, Love JM, McAleer MA, Prins JB, Rodrigues N, Lathrop M, Pressey A, Delarato NH, Peterson LB, Wicker LS 1991 Genetic analysis of autoimmune type 1 diabetes mellitus in mice. Nature 351:542–547
52. Wicker LS, Appel MC, Dotta F, Pressey A, Miller BJ, Delarato NH, Fischer PA, Boltz Jr RC, Peterson LB 1992 Autoimmune syndromes in major histocompatibility complex (MHC) congenic strains of nonobese diabetic (NOD) mice. The NOD MHC is dominant for insulitis and cyclophosphamide-induced diabetes. J Exp Med 176:67–77
53. Slattery RM, Kjer-Nielsen L, Allison J, Charlton B, Mandel TE, Miller JF 1990 Prevention of diabetes in non-obese diabetic I-Ak transgenic mice. Nature 345:724–726
54. Miyazaki T, Uno M, Uehira M, Kikutani H, Kishimoto T, Kimoto M, Nishimoto H, Miyazaki J, Yamamura K 1990 Direct evidence for the contribution of the unique I-AND to the development of insulitis in non-obese diabetic mice. Nature 345:722–724
55. Lund T, O'reilly L, Hutchings P, Kanagawa O, Simpson E, Gravely R, Chandler P, Dyson J, Picard JK, Edwards A, Kioussis D, Cooke A 1990 Prevention of insulin-dependent diabetes mellitus in non-obese diabetic mice by transgenes encoding modified I-A beta-chain or normal I-E alpha-chain. Nature 345:727–729
56. Nishimoto H, Kikutani H, Yamamura K, Kishimoto T 1987 Prevention of autoimmune insulitis by expression of I-E molecules in NOD mice. Nature 328:432–434
57. Bohme J, Schulzbaur B, Kanagawa O, Benoist C, Mathis D 1990 MHC-linked protection from diabetes dissociated from clonal deletion of T cells. Science 249:293–295
58. Miyazaki T, Matsuda Y, Toyonaga T, Miyazaki J, Yazaki Y, Yamamura K 1992 Prevention of autoimmune insulitis in non-obese diabetic mice by expression of major histocompatibility complex class I Ld molecules. Proc Natl Acad Sci USA 89:9519–9523
59. Hattori M, Buse JB, Jackson RA, Glimcher L, Dorf ME, Minami M, Makino S, Moriwaki K, Kuzuya H, Imura H, Strauss WM, Seidman JG, Eisenbarth GS 1986 The NOD mouse: recessive diabetogenic gene in the major histocompatibility complex. Science 231:733–735
60. Acha-Orbea H, McDevitt HO 1987 The first external domain of the nonobese diabetic mouse class II I-A beta chain is unique. Proc Natl Acad Sci USA 84:2435–2439
61. Podolin PL, Pressey A, Delarato NH, Fischer PA, Peterson LB, Wicker LS 1993 I-E+ nonobese diabetic mice develop insulitis and diabetes. J Exp Med 178:793–803
62. Colle E, Guttmann RD, Seemayer T 1981 Spontaneous diabetes mellitus syndrome in the rat. I. Association with the major histocompatibility complex. J Exp Med 154:1237–1242
63. Colle E, Guttmann RD, Seemayer TA 1985 Association of spontaneous thyroiditis with the major histocompatibility complex of the rat. Endocrinology 116:1243–1247
64. Wassmuth R, Lernmark A 1989 The genetics of susceptibility to diabetes. Clin Immunol Immunopathol 53:358–399
65. Thorsby E, Ronningen KS 1993 Particular HLA-DQ molecules play a dominant role in determining susceptibility or resistance to type 1 (insulin-dependent) diabetes mellitus. Diabetologia 36:371–377
66. Baisch JM, Weeks T, Giles R, Hoover M, Stastny P, Capra JD 1990 Analysis of HLA-DQ genotypes and susceptibility in insulin-dependent diabetes mellitus. N Engl J Med 322:1836–1841
67. Caillat-Zucman S, Garchon HJ, Timsit J, Assan R, Boitard C, Djilali-Salah I, Bougneres P, Bach JF 1992 Age-dependent HLA genetic heterogeneity of type 1 insulin-dependent diabetes mellitus. J Clin Invest 90:2242–2250
68. Todd JA, Bell JI, McDevitt HO 1987 HLA-DQ beta gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. Nature 329:599–604
69. Charron DJ, Lotteau V, Turmel P 1984 Hybrid HLA-DC antigens provide molecular evidence for gene trans-complementation. Nature 312:157–159
70. Ronningen KS, Undlien DE, Ploski R, Maouni N, Konrad RJ, Jensen E, Hornes E, Reijonen H, Colonna M, Monos DS, Strominger JL, Thorsby E 1993 Linkage disequilibrium between

TAP2 variants and HLA class II alleles; no primary association between TAP2 variants and insulin-dependent diabetes mellitus. Eur J Immunol 23:1050–1056
71. Caillat-Zucman S, Bertin E, Timsit J, Boitard C, Assan R, Bach JF 1993 Protection from insulin-dependent diabetes mellitus is linked to a peptide transporter gene. Eur J Immunol 23:1784–1788
72. Bell GI, Horita S, Karam JH 1984 A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. Diabetes 33:176–183
73. Julier C, Hyer RN, Davies J, Merlin F, Soularue P, Briant L, Cathelineau G, Deschamps I, Rotter JI, Froguel P, Boitard C, Bell JI, Lathrop GM 1991 Insulin-IGF2 region on chromosome 11p encodes a gene implicated in HLA-DR4-dependent diabetes susceptibility. Nature 354:155–159
74. Bain SC, Prins JB, Hearne CM, Rodrigues NR, Rowe BR, Pritchard LE, Ritchie RJ, Hall JR, Undlien DE, Ronningen KS, Dunger DB, Barnett AH, Todd JA 1992 Insulin gene region-encoded susceptibility to type 1 diabetes is not restricted to HLA-DR4-positive individuals. Nat Genet 2:212–215
75. Lucassen AM, Julier C, Beressi JP, Boitard C, Froguel P, Lathrop M, Bell JI 1993 Susceptibility to insulin dependent diabetes mellitus maps to a 4.1 kb segment of DNA spanning the insulin gene and associated VNTR. Nat Genet 4:305–310
76. Avoustin P, Briant L, de Preval C, Cambon-Thomsen A 1992 Polymorphism study of TCR alpha and gamma genes in insulin dependent diabetes mellitus (IDDM) multiplex families. Autoimmunity 14:97–100
77. Martinez-Naves E, Coto E, Gutierrez V, Urra JM, Setien F, Dominguez O, Hood LE, Lopez-Larrea C 1991 Germline repertoire of T-cell receptor beta-chain genes in patients with insulin-dependent diabetes mellitus. Hum Immunol 31:77–80
78. Kelly H, Garlepp MJ 1993 T cell receptor haplotypes in families of patients with insulin-dependent diabetes mellitus. Clin Exp Immunol 91:226–231
79. Cornall RJ, Prins JB, Todd JA, Pressey A, Delarato NH, Wicker LS, Peterson LB 1991 Type 1 diabetes in mice is linked to the interleukin-1 receptor and Lsh/Ity/Bcg genes on chromosome 1. Nature 353:262–265
80. Garchon HJ, Bedossa P, Eloy L, Bach JF 1991 Identification and mapping to chromosome 1 of a susceptibility locus for periinsulitis in non-obese diabetic mice. Nature 353:260–262
81. Ghosh S, Palmer SM, Rodrigues NR, Cordell HJ, Hearne CM, Cornall RJ, Prins JB, McShane P, Lathrop GM, Peterson LB, Wicker LS, Todd JA 1993 Polygenic control of autoimmune diabetes in nonobese diabetic mice. Nat Genet 4:404–409
82. De Gouyon B, Melanitou E, Richard MF, Requarth M, Hahn IH, Guenet JL, Demenais F, Julier C, Lathrop GM, Boitard C, Avner P 1993 Genetic analysis of diabetes and insulitis in an interspecific cross of the nonobese diabetic mouse with Mus spretus. Proc Natl Acad Sci USA 90:1877–1881
83. Prins JB, Todd JA, Rodrigues NR, Ghosh S, Hogarth PM, Wicker LS, Gaffney E, Podolin PL, Fischer PA, Sirotina A, Peterson LB 1993 Linkage on chromosome 3 of autoimmune diabetes and defective Fc receptor for IgG in NOD mice. Science 260:695–698
84. Garchon HJ, Luan JJ, Eloy L, Bédossa P, Bach JF 1994 Genetic analysis of immune dysfunction in non-obese diabetic (NOD) mice: mapping of a susceptibility locus close to the Bcl-2 gene correlates with increased resistance of NOD T cells to apoptosis induction. Eur J Immunol 24:380–384
85. Jacob HJ, Pettersson A, Wilson D, Mao Y, Lernmark A, Lander ES 1992 Genetic dissection of autoimmune type 1 diabetes in the BB rat. Nat Genet 2:56–60
86. Diabetes Epidemiology Research International Group 1988 Geographic patterns of childhood insulin-dependent diabetes mellitus. Diabetes 37:1113–1119
87. Patrick SL, Moy CS, Laporte RE 1989 The world of insulin-dependent diabetes mellitus: what international epidemiologic studies reveal about the etiology and natural history of IDDM. Diabetes Metab Rev 5:571–578
88. Lefkowith J, Schreiner G, Cormier J, Handler ES, Driscoll HK, Greiner D, Mordes JP, Rossini AA 1990 Prevention of diabetes in the BB rat by essential fatty acid deficiency. Relationship between physiological and biochemical changes. J Exp Med 171:729–743
89. Elliott RB, Reddy SN, Bibby NJ, Kida K 1988 Dietary prevention of diabetes in the non-obese diabetic mouse. Diabetologia 31:62–64
90. Issa-Chergui B, Guttmann RD, Seemayer TA, Kelley VE, Colle E 1988 The effect of diet on the spontaneous insulin dependent diabetic syndrome in the rat. Diabetes Res 9:81–86
91. Oldstone MB 1990 Viruses as therapeutic agents. I. Treatment of nonobese insulin-dependent diabetes mice with virus prevents insulin-dependent diabetes mellitus while maintaining general immune competence. J Exp Med 171:2077–2089
92. Oldstone MB, Ahmed R, Salvato M 1990 Viruses as therapeutic agents. II. Viral reassortants map prevention of insulin-dependent diabetes mellitus to the small RNA of lymphocytic choriomeningitis virus. J Exp Med 171:2091–2100
93. Takei I, Asaba Y, Kasatani T, Maruyama T, Watanabe K, Yanagawa T, Saruta T, Ishii T 1992 Suppression of development of diabetes in NOD mice by lactate dehydrogenase virus infection. J Autoimmun 5:665–673
94. Wilberz S, Partke HJ, Dagnaes-Hansen F, Herberg L 1991 Persistent MHV (mouse hepatitis virus) infection reduces the incidence of diabetes mellitus in non-obese diabetic mice. Diabetologia 34:2–5
95. Like AA, Guberski DL, Butler L 1991 Influence of environmental viral agents on frequency and tempo of diabetes mellitus in BB/Wor rats. Diabetes 40:259–262
95a. Bach JF 1994 Predictive medicine in autoimmune diseases: from the identification of genetic predisposition and environmental influence to precocious immunotherapy. Clin Immunol Immunopathol, in press
96. Guberski DL, Thomas VA, Shek WR, Like AA, Handler ES, Rossini AA, Wallace JE, Welsh RM 1991 Induction of type 1 diabetes by Kilham's rat virus in diabetes-resistant BB/Wor rats. Science 254:1010–1013
97. Elliott RB, Martin JM 1984 Dietary protein: a trigger of insulin-dependent diabetes in the BB rat? Diabetologia 26:297–299
98. Daneman D, Fishman L, Clarson C, Martin JM 1987 Dietary triggers of insulin-dependent diabetes in the BB rat. Diabetes Res 5:93–97
99. Laakso M, Rounanen A, Klaukka T, Aromaa A, Maatela J, Pyorala K 1991 Changes in the prevalence and incidence of diabetes mellitus in Finnish adults, 1970–1987. Am J Epidemiol 133:850–857
100. Songini M, Muntoni S 1991 High incidence of type-I diabetes in Sardinia. Lancet 337:1047
101. Yoon JW, Ihn SH 1990 Viruses as a triggering factor of autoimmune type 1 diabetes. In Farid NR, Bona CA (eds) The Molecular Aspects of Autoimmunity. Academic Press, London, pp 231–240
102. Yoon JW 1991 Role of viruses in the pathogenesis of IDDM. Ann Med 23:437–445
103. Macdonald MJ, Liston L, Carlson I 1987 Seasonality in glycosylated hemoglobin in normal subjects. Does seasonal incidence in insulin-dependent diabetes suggest specific etiology? Diabetes 36:265–268
104. Menser MA, Forrest JM, Bransby RD 1978 Rubella infection and diabetes mellitus. Lancet 1:57–60
105. Yoon JW, Austin M, Onodera T, Notkins AL 1979 Virus induced diabetes mellitus. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med 300:1173–1179
106. King ML, Shaikh A, Bidwell D, Voller A, Banatvala JE 1983 Coxsackie-B-virus-specific IgM responses in children with insulin-dependent (juvenile-onset; type I) diabetes mellitus. Lancet 1:1397–1399
107. Craighead JE, McLane MF 1968 Diabetes mellitus: induction in mice by encephalomyocarditis virus. Science 162:913–914
108. Haynes MK, Huber SA, Craighead JE 1987 Helper-inducer T-lymphocytes mediate diabetes in EMC-infected BALB/c ByJ mice. Diabetes 36:877–881
109. Buschard K, Rygaard J, Lung E 1976 The inability of a diabetogenic virus to induce diabetes mellitus in athymic (nude) mice. Acta Pathol Microbiol Scand C 84:299–303
110. Rayfield EJ, Kelly KJ, Yoon JW 1986 Rubella virus-induced diabetes in the hamster. Diabetes 35:1278–1281
111. Gaskins HR, Prochazka M, Hamaguchi K, Serreze DV, Leiter EH 1992 Beta cell expression of endogenous xenotropic retrovirus distinguishes diabetes-susceptible NOD/Lt from resistant NON/Lt mice. J Clin Invest 90:2220–2227

112. Suenaga K, Yoon JW 1988 Association of beta-cell-specific expression of endogenous retrovirus with development of insulitis and diabetes in NOD mouse. Diabetes 37:1722–1726
113. Thomas VA, Woda BA, Handler ES, Greiner DL, Mordes JP, Rossini AA 1991 Altered expression of diabetes in BB/Wor rats by exposure to viral pathogens. Diabetes 40:255–258
114. Kaufman DL, Erlander MG, Clare-Salzler M, Atkinson MA, MacLaren NK, Tobin AJ 1992 Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. J Clin Invest 89:283–292
115. Sibley RK, Sutherland DE, Goetz F, Michael AF 1985 Recurrent diabetes mellitus in the pancreas iso- and allograft. A light and electron microscopic and immunohistochemical analysis of four cases. Lab Invest 53:132–144
116. Lampeter EF, Homberg M, Quabeck K, Schaefer UW, Wernet P, Bertrams J, Grosse-Wilde H, Gries FA, Kolb H 1993 Transfer of insulin-dependent diabetes between HLA-identical siblings by bone marrow transplantation. Lancet 341:1243–1244
117. Viallettes B, Mancinelli D 1993 Transfer of insulin-dependent diabetes between HLA-identical siblings by bone marrow transplantation. Lancet 342:174
118. Wang Y, Pontesilli O, Gill RG, La Rosa FG, Lafferty KJ 1991 The role of CD4+ and CD8+ T cells in the destruction of islet grafts by spontaneously diabetic mice. Proc Natl Acad Sci USA 88:527–531
119. Weringer EJ, Like AA 1985 Immune attack on pancreatic islet transplants in the spontaneously diabetic BioBreeding/Worcester (BB/W) rat is not MHC restricted. J Immunol 134:2383–2386
120. Sadelain MW, Qin HY, Lauzon J, Singh B 1990 Prevention of type I diabetes in NOD mice by adjuvant immunotherapy. Diabetes 39:583–589
121. McInerney MF, Pek SB, Thomas DW 1991 Prevention of insulitis and diabetes onset by treatment with complete Freund's adjuvant in NOD mice. Diabetes 40:715–725
122. Sadelain MW, Qin HY, Sumoski W, Parfrey N, Singh B, Rabinovitch A 1990 Prevention of diabetes in the BB rat by early immunotherapy using Freund's adjuvant. J Autoimmun 3: 671–680
123. Qin HY, Suarez WL, Parfrey N, Power RF, Rabinovitch A 1992 Mechanisms of complete Freund's adjuvant protection against diabetes in BB rats: induction of non-specific suppressor cells. Autoimmunity 12:193–199
124. Yagi H, Matsumoto M, Kishimoto Y, Makino S, Harada M 1991 Possible mechanism of the preventive effect of BCG against diabetes mellitus in NOD mouse. II. Suppression of pathogenesis by macrophage transfer from BCG-vaccinated mice. Cell Immunol 138:142–149
124a. Shehadeh N, Calcinaro F, Bradley BJ, Bruchilm I, Vardi P, Lafferty KJ 1994 Effect of adjuvant therapy on development of diabetes in mouse and man. Lancet 343:706–707
125. Renold AE 1985 Possible animal models for diabetes mellitus: syndromes involving toxic or immune etiology. In: Alberti KG, Krall LP (eds) The Diabetes Annual. Elsevier, Amsterdam, pp 492–508
126. Karam JH, Lewitt PA, Young CW, Nowlain RE, Frankel BJ, Fujiya H, Freedman ZR, Grodsky GM 1980 Insulinopenic diabetes after rodenticide (Vacor) ingestion: a unique model of acquired diabetes in man. Diabetes 29:971–978
127. Bouchard P, Sai P, Reach G, Caubarrere I, Ganeval D, Assan R 1982 Diabetes mellitus following pentamidine-induced hypoglycemia in humans. Diabetes 31:40–45
128. Karjalainen J, Martin JM, Knip M, Ilonen J, Robinson BH, Savilahti E, Akerblom HK, Dosch HM 1992 A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. N Engl J Med 327:302–307
129. Robinson BH, Dosch HM, Martin JM, Akerblom HK, Savilahti E, Knip M, Ilonen J 1993 A model for the involvement of MHC class II proteins in the development of type 1 (insulin-dependent) diabetes mellitus in response to bovine serum albumin peptides. Diabetologia 36:364–368
130. Dahlquist G, Savilahti E, Landin-Olsson M 1992 An increased level of antibodies to beta-lactoglobulin is a risk determinant for early-onset type 1 (insulin-dependent) diabetes mellitus independent of islet cell antibodies and early introduction of cow's milk. Diabetologia 35:980–984
131. Karjalainen J, Saukkonen T, Savilahti E, Dosch HM 1992 Disease-associated anti-bovine serum albumin antibodies in type 1 (insulin-dependent) diabetes mellitus are detected by particle concentration fluoroimmunoassay, and not by enzyme linked immunoassay. Diabetologia 35:985–990
132. Savilahti E, Saukkonen TT, Virtala ET, Tuomilehto J, Akerblom HK 1993 Increased levels of cow's milk and beta-lactoglobulin antibodies in young children with newly diagnosed IDDM. Diabetes Care 16:984–989
133. Atkinson MA, Bowman MA, Kao KJ, Campbell L, Dush PJ, Shah SC, Simell O, MacLaren NK 1993 Lack of immune responsiveness to bovine serum albumin in insulin-dependent diabetes. N Engl J Med 329:1853–1858
134. Pietropaolo M, Castano L, Babu S, Buelow R, Kuo YL, Martin S, Martin A, Powers AC, Prochazka M, Naggert J, Leiter EH, Eisenbarth GS 1993 Islet cell autoantigen 69 kD (ICA69). Molecular cloning and characterization of a novel diabetes-associated autoantigen. J Clin Invest 92:359–371
135. Surwit RS, Schneider MS, Feinglos MN 1992 Stress and diabetes mellitus. Diabetes Care 15:1413–1422
136. Ader DN, Johnson SB, Huang SW, Riley WJ 1991 Group size, cage shelf level, and emotionality in non-obese diabetic mice: impact on onset and incidence of IDDM. Psychosom Med 53: 313–321
137. Durant S, Coulaud J, Amrani A, El Hasnaoui A, Dardenne M, Homo-Delarche F 1993 Effects of various environmental stress paradigms and adrenalectomy on the expression of autoimmune type 1 diabetes in the nonobese diabetic (NOD) mouse. J Autoimmun 6:735–751
138. Williams AJ, Krug J, Lampeter EF, Mansfield K, Beales PE, Signore A, Gale EA, Pozzilli P 1990 Raised temperature reduces the incidence of diabetes in the NOD mouse. Diabetologia 33: 635–637
139. Fitzpatrick F, Lepault F, Homo-Delarche F, Bach JF, Dardenne M 1991 Influence of castration, alone or combined with thymectomy, on the development of diabetes in the nonobese diabetic mouse. Endocrinology 129:1382–1390
140. Fox HS 1992 Androgen treatment prevents diabetes in nonobese diabetic mice. J Exp Med 175:1409–1412
141. Rose NR, Bona C 1993 Defining criteria for autoimmune diseases (Witebsky's postulates revisited). Immunol Today 14:426–430
141a. Gaedigk R, Duncan AMV, Miyazaki I, Robinson BH, Dosch HM 1994 ICA1 encoding p69, a protein linked to the development of type 1 diabetes, maps to human chromosome 7p22. Cytogenet Cell Genet 66:274–276
142. Bendelac A, Carnaud C, Boitard C, Bach JF 1987 Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4+ and Lyt-2+ T cells. J Exp Med 166:823–832
143. Koevary S, Rossini A, Stoller W, Chick W, Williams RM 1983 Passive transfer of diabetes in the BB/W rat. Science 220:727–728
144. Haskins K, McDuffie M 1990 Acceleration of diabetes in young NOD mice with a CD4+ Islet-specific T cell clone. Science 249:1433–1436
145. Wicker LS, Miller BJ, Mullen Y 1986 Transfer of autoimmune diabetes mellitus with splenocytes from nonobese diabetic (NOD) mice. Diabetes 35:855–860
146. Shimizu J, Kanagawa O, Unanue ER 1993 Presentation of beta-cell antigens to CD4+ and CD8+ T cells of non-obese diabetic mice. J Immunol 151:1723–1730
147. Feutren G, Papoz L, Assan R, Vialettes B, Karsenty G, Vexiau P, Du Rostu H, Rodier M, Sirmai J, Lallemand A, Bach JF 1986 Cyclosporin increases the rate and length of remissions in insulin-dependent diabetes of recent onset. Results of a multicentre double-blind trial. Lancet 2:119–124
148. The Canadian-European Randomized Control Trial Group 1988 Cyclosporin-induced remission of IDDM after early intervention. Association of 1 yr of cyclosporin treatment with enhanced insulin secretion. Diabetes 37:1574–1582
149. Bach JF 1993 Strategies in immunotherapy of insulin-dependent diabetes mellitus. Ann NY Acad Sci, in press
150. Bottazzo GF, Florin-Christensen A, Doniach D 1974 Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. Lancet 2:1279–1283
151. Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M,

Cascalho M, Folli F, Richter-Olesen H, de Camilli P 1990 Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 347:151–156

152. Atkinson MA, Kaufman DL, Campbell L, Gibbs KA, Shah SC, Bu DF, Erlander MG, Tobin AJ, MacLaren NK 1992 Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. Lancet 339:458–459
153. Honeyman MC, Cram DS, Harrison LC 1993 Glutamic acid decarboxylase 67-reactive T cells: a marker of insulin-dependent diabetes. J Exp Med 177:535–540
154. Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, Paquette TL 1983 Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science 222:1337–1339
155. Kuglin B, Halder B, Bertrams J, Grunekleo D, Gries FA, Kolb H 1990 Proinsulin autoantibodies: association with type 1 diabetes but not with islet cell antibodies, insulin autoantibodies or HLA-DR type. J Autoimmun 3:573–577
156. Cabrera E, Fernandez LE, Carr A, Marquina G, Valiente O, Uriarte A, Rynmark BM, Fredman P, Svennerholm L 1992 Which glycolipids are the autoantigens of cytoplasmic islet cell antibodies ? Acta Diabetol 29:70–74
157. Genovese S, Bonifacio E, McNally JM, Dean BM, Wagner R, Bosi E, Gale EA, Bottazzo GF 1992 Distinct cytoplasmic islet cell antibodies with different risks for type 1 (insulin-dependent) diabetes mellitus. Diabetologia 35:385–388
158. Dobersen MJ, Scharff JE, Ginsberg-Fellner F, Notkins AL 1980 Cytotoxic autoantibodies to beta cells in the serum of patients with insulin-dependent diabetes mellitus. N Engl J Med 303:1493–1498
159. Sai P, Boitard C, Debray-Sachs M, Fouplard A, Assan R, Hamburger J 1981 Complement-fixing islet cell antibodies from some diabetic patients alter insulin release *in vitro*. Diabetes 30: 1051–1057
160. Nerup J, Andersen OO, Bendixen G, Egeberg J, Poulsen JE 1971 Antipancreatic cellular hypersensitivity in diabetes mellitus. Diabetes 20:424–427
161. Harrison LC, de Aizpurua H, Loudovaris T, Campbell IL, Cebon JS, Tait BD, Colman PG 1991 Reactivity to human islets and fetal pig proislets by peripheral blood mononuclear cells from subjects with preclinical and clinical insulin-dependent diabetes. Diabetes 40:1128–1133
162. Harrison LC, Chu SX, de Aizpurua HJ, Graham M, Honeyman MC, Colman PG 1992 Islet-reactive T cells are a marker of preclinical insulin-dependent diabetes. J Clin Invest 89:1161–1165
163. Miller BJ, Appel MC, O'neil JJ, Wicker LS 1988 Both the Lyt-2+ and L3T4+ T cell subsets are required for the transfer of diabetes in nonobese diabetic mice. J Immunol 140:52–58
164. Edouard P, Hiserodt JC, Flamondon C, Poussier P 1993 CD8+ T-cells are required for adoptive transfer of the BB rat diabetic syndrome. Diabetes 42:390–397
165. Kloppel G, Altenahr E, Freytag G, Jansen FK 1974 Immune insulitis and manifest diabetes mellitus. Studies on the course of immune insulitis and the induction of diabetes mellitus in rabbits immunized with insulin. Virchows Arch [A] 364:333–346
166. Geenen V, Kroemer G 1993 Multiple ways to cellular immune tolerance. Immunol Today 14:573–575
167. Kaufman DL, Clare-Salzler M, Tian J, Forsthuber T, Ting GS, Robinson P, Atkinson MA, Sercarz EE, Tobin AJ, Lehmann PV 1993 Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. Nature 366:69–72
168. Tisch R, Yang XD, Singer SM, Liblau RS, Fugger L, McDevitt HO 1993 Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. Nature 366:72–75
169. Gepts W, Lecompte PM 1981 The pancreatic islets in diabetes. Am J Med 70:105–115
170. Bottazzo GF, Dean BM, McNally JM, MacKay EH, Swift PG, Gamble DR 1985 *In situ* characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. N Engl J Med 313:353–360
171. Hanninen A, Jalkanen S, Salmi M, Toikkanen S, Nikolakaros G, Simell O 1992 Macrophages, T cell receptor usage, and endothelial cell activation in the pancreas at the onset of insulin-dependent diabetes mellitus. J Clin Invest 90:1901–1910
172. Karlsson-Parra A, Kobbah M, Ewald U, Tuvemo T, Porsum U, Klareskog L 1988 Circulating class II transplantation antigen-expressing T lymphocytes in children with insulin-dependent diabetes mellitus at diagnosis. Acta Paediatr Scand 77:554–558
173. Chatenoud L, Feutren G, Nelson DL, Boitard C, Bach JF 1989 Effect of cyclosporin on interleukin 2-related T-lymphocyte parameters in IDDM patients. Diabetes 38:249–256
174. Giordano C, Galluzzo A, Marco A, Panto F, Amato MP, Caruso C, Bompiani GD 1988 Increased soluble interleukin-2 receptor levels in the sera of type 1 diabetic patients. Diabetes Res 8: 135–138
175. Munoz A, Gallart T, Vinas O, Gomis R 1991 Increased CD5-positive B lymphocytes in type 1 diabetes. Clin Exp Immunol 83:304–308
176. MacKay P, Jacobson J, Rabinovitch A 1986 Spontaneous diabetes mellitus in the Bio-Breeding/Worcester rat. Evidence *in vitro* for natural-killer cell lysis of islet cells. J Clin Invest 77:916–924
177. Rapoport MJ, Lazarus AH, Jaramillo A, Speck E, Delovitch TL 1993 Thymic T cell anergy in autoimmune nonobese diabetic mice is mediated by deficient T cell receptor regulation of the pathway of p21ras activation. J Exp Med 177:1221–1226
178. Seape P, Ezine S, Marvel J, Bedossa P, Richard MF, Bach JF, Boitard C 1993 Role of CD4+CD45RA+ T cells in the development of autoimmune diabetes in the non-obese diabetic (NOD) mouse. Int Immunol 5:479–489
179. Savino W, Boitard C, Bach JF, Dardenne M 1991 Studies on the thymus in nonobese diabetic mouse. 1. Changes in the microenvironmental compartments. Lab Invest 64:405–417
180. Savino W, Carnaud C, Luan JJ, Bach JF, Dardenne M 1993 Characterization of the extracellular matrix-containing giant perivascular spaces in the NOD mouse thymus. Diabetes 42:134–140
181. Nabarra B, Andrianarison I 1991 Thymus reticulum of autoimmune mice. 3. Ultrastructural study of NOD (non-obese diabetic) mouse thymus. Int J Exp Pathol 72:275–287
182. Rozing J, Coolen C, Tielen FJ, Weegenaar J, Schuurman HJ, Greiner DL, Rossini AA 1989 Defects in the thymic epithelial stroma of diabetes prone BB rats. Thymus 14:125–135
183. Rapoport MJ, Jaramillo A, Zipris D, Lazarus AH, Serreze DV, Leiter EH, Cyopick P, Danska JS, Delovitch TL 1993 Interleukin 4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice. J Exp Med 178:87–99
184. Boitard C, Larger E, Timsit J, Bach JF 1994 Insulin-dependent diabetes mellitus: an islet or an immune disease? Diabetologia, in press
185. Tron F, Bach JF 1989 Molecular and genetic characteristics of pathogenic autoantibodies. J Autoimmun 2:311–320
186. Pontes de Carvalho LC, Templeman J, Wick G, Roitt IM 1982 The role of self-antigen in the development of autoimmunity in Obese strain chickens with spontaneous autoallergic thyroiditis. J Exp Med 155:1255–1266
187. Berzofsky JA, Berkower IJ 1989 Immunogenicity and antigen structure. In: Paul WE (ed) Fundamental Immunology, ed 2. Raven Press, New York, vol 3:169–208
188. Baekkeskov S, Landin M, Kristensen JK, Srikanta S, Brulning GJ, Mandrup-Poulsen T, de Beaufort C, Sneldner JS, Eisenbarth G, Lindgren F, Sundquist G, Lernmark A 1987 Antibodies to a 64,000 Mr human islet cell antigen precede the clinical onset of insulin-dependent diabetes. J Clin Invest 79:926–934
189. Bu DF, Erlander MG, Hitz BC, Tillakaratne NJ, Kaufman DL, Wagner-McPherson CB, Evans GA, Tobin AJ 1992 Two human glutamate decarboxylases, 65-kDa GAD and 67-kDa GAD, are each encoded by a single gene. Proc Natl Acad Sci USA 89: 2115–2119
190. Karlsen AE, Hagopian WA, Grubin CE, Dube S, Disteche CM, Adler DA, Barmeier H, Mathewes S, Grant FJ, Foster D, Lernmark A 1991 Cloning and primary structure of a human islet isoform of glutamic acid decarboxylase from chromosome 10. Proc Natl Acad Sci USA 88:8337–8341
191. Hagopian WA, Karlsen AE, Gottsater A, Landin-Olsson M, Grubin CE, Sundkvist G, Petersen JS, Boel E, Dyrberg T, Lernmark A 1993 Quantitative assay using recombinant human islet glutamic acid decarboxylase (GAD65) shows that 64K autoantibody positivity at onset predicts diabetes type. J Clin Invest 91: 368–374
192. Hagopian WA, Michelsen B, Karlsen AE, Larsen F, Moody A, Grubin CE, Rowe R, Petersen J, McEvoy R, Lernmark A 1993 Autoantibodies in IDDM primarily recognize the 65,000-M(r)

rather than the 67,000-M(r) isoform of glutamic acid decarboxylase. Diabetes 42:631–636
193. Atkinson MA, Kaufman DL, Newman D, Tobin AJ, MacLaren NK 1993 Islet cell cytoplasmic autoantibody reactivity to glutamate decarboxylase in insulin-dependent diabetes. J Clin Invest 91: 350–356
194. Harrison LC, Honeyman MC, de Aizpurua HJ, Schmidli RS, Colman PG, Tait BD, Cram DS 1993 Inverse relation between humoral and cellular immunity to glutamic acid decarboxylase in subjects at risk of insulin-dependent diabetes. Lancet 341: 1365–1369
195. Thivolet CH, Tappaz M, Durand A, Petersen J, Stefanutti A, Chatelain P, Vialettes B, Scherbaum W, Orgiazzi J 1992 Glutamic acid decarboxylase (GAD) autoantibodies are additional predictive markers of type 1 (insulin-dependent) diabetes mellitus in high risk individuals. Diabetologia 35:570–576
196. Christie MR, Tun RY, Lo SS, Cassidy D, Brown TJ, Hollands J, Shattock M, Bottazzo GF, Leslie RD 1992 Antibodies to GAD and tryptic fragments of islet 64K antigen as distinct markers for development of IDDM. Studies with identical twins. Diabetes 41:782–787
197. Christie MR, Vohra G, Champagne P, Daneman D, Delovitch TL 1990 Distinct antibody specificities to a 64-kD islet cell antigen in type 1 diabetes as revealed by trypsin treatment. J Exp Med 172:789–794
198. Christie MR, Hollands JA, Brown TJ, Michelsen BK, Delovitch TL 1993 Detection of pancreatic islet 64,000 M(r) autoantigens in insulin-dependent diabetes distinct from glutamate decarboxylase. J Clin Invest 92:240–248
199. Muir A, Luchetta R, Song HY, Peck A, Krischer J, MacLaren N 1993 Insulin immunization protects NOD mice from diabetes. Autoimmunity 15:58 (Abstract)
200. Zhang ZJ, Davidson L, Eisenbarth G, Weiner HL 1991 Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. Proc Natl Acad Sci USA 88:10252–10256
201. Atkinson MA, MacLaren NK, Luchetta R 1990 Insulitis and diabetes in NOD mice reduced by prophylactic insulin therapy. Diabetes 39:933–937
202. Thivolet CH, Goillot E, Bedossa P, Durand A, Bonnard M, Orgiazzi J 1991 Insulin prevents adoptive cell transfer of diabetes in the autoimmune non-obese diabetic mouse. Diabetologia 34:314–319
203. Gottfreden CF, Buschard K, Frandsen EK 1985 Reduction of diabetes incidence of BB Wistar rats by early prophylactic insulin treatment of diabetes-prone animals. Diabetologia 28:933–935
204. Bertrand S, de Paepe M, Vigeant C, Yale JF 1992 Prevention of adoptive transfer in BB rats by prophylactic insulin treatment. Diabetes 41:1273–1277
205. Gottlieb PA, Handler ES, Appel MC, Greiner DL, Mordes JP, Rossini AA 1991 Insulin treatment prevents diabetes mellitus but not thyroiditis in RT6-depleted diabetes resistant BB/Wor rats. Diabetologia 34:296–300
206. Keller RJ, Eisenbarth GS, Jackson RA 1993 Insulin prophylaxis in individuals at high risk of type I diabetes. Lancet 341:927–928
206a. Elias D, Cohen IR 1994 Peptide therapy for diabetes in NOD mice. Lancet 343:704–706
207. Roep BO, Arden SD, de Vries RR, Hutton JC 1990 T-cell clones from a type-1 diabetes patient respond to insulin secretory granule proteins. Nature 345:632–634
208. Roep BO, Kallan AA, Hazenbos WL, Bruining GJ, Bailyes EM, Arden SD, Hutton JC, de Vries RR 1991 T-cell reactivity to 38 kD insulin-secretory-granule protein in patients with recent-onset type 1 diabetes. Lancet 337:1439–1441
209. Boitard C, Villa MC, Becourt C, Gia HP, Huc C, Sempe P, Portier MM, Bach JF 1992 Peripherin: an islet antigen that is cross-reactive with nonobese diabetic mouse class II gene products. Proc Natl Acad Sci USA 89:172–176
210. Castano L, Russo E, Zhou L, Lipes MA, Eisenbarth GS 1991 Identification and cloning of a granule autoantigen (carboxypeptidase-H) associated with type I diabetes. J Clin Endocrinol Metab 73:1197–1201
211. Nossal GJ, Herold KC, Goodnow CC 1992 Autoimmune tolerance and type 1 (insulin-dependent) diabetes mellitus. Diabetologia 35 (Suppl 2):S49–S59
212. Shehadeh NN, Gill RG, Lafferty KJ 1993 Mechanism of self-tolerance to endocrine tissue. Springer Semin Immunopathol 14:203–220
213. Ametani A, Sercarz EE 1993 The nature of B- and T-cell determinants. In: Bach JF (ed) Monoclonal Antibodies and Peptide Therapy in Autoimmune Diseases. Marcel Dekker, New York, vol:13–28
214. Nakano N, Kikutani H, Nishimoto H, Kishimoto T 1991 T cell receptor V gene usage of islet beta cell-reactive T cells is not restricted in non-obese diabetic mice. J Exp Med 173:1091–1097
215. Katz JD, Wang B, Haskins K, Benoist C, Mathis D 1993 Following a diabetogenic T cell from genesis through pathogenesis. Cell 74:1089–1100
216. Acha-Orbea H 1993 T-cell receptors in autoimmune disease. In: Bach JF (ed) Monoclonal Antibodies and Peptide Therapy in Autoimmune Diseases. Marcel Dekker, New York, vol:131–142
217. O'reilly LA, Hutchings PR, Crocker PR, Simpson E, Lund T, Kioussis D, Takei F, Baird J, Cooke A 1991 Characterization of pancreatic islet cell infiltrates in NOD mice: effect of cell transfer and transgene expression. Eur J Immunol 21:1171–1180
218. Toyoda H, Redford A, Magalong D, Chan E, Hosszufalusi N, Formby B, Teruya M, Charles MA 1992 *In situ* islet T cell receptor variable region gene usage in the nonobese diabetic mouse. Immunol Lett 32:241–245
219. Candas S, Katz J, Benoist C, Mathis D, Haskins K 1991 Islet-specific T-cell clones from nonobese diabetic mice express heterogeneous T-cell receptors. Proc Natl Acad Sci USA 88:6167–6170
220. Bacelj A, Charlton B, Mandel TE 1989 Prevention of cyclophosphamide-induced diabetes by anti-V beta 8 T-lymphocyte-receptor monoclonal antibody therapy in NOD/Wehi mice. Diabetes 38:1492–1495
221. Taki T, Yokono K, Amano K, Hatamori N, Hirao Y, Tominaga Y, Maeda S, Kasuga M 1993 Effect of T-cell receptor V beta-specific monoclonal antibodies on cyclophosphamide-induced diabetes mellitus in non-obese diabetic mice. Diabetologia 36: 391–396
222. Edouard P, Thivolet C, Bedossa P, Olivi M, Legrand B, Bendelac A, Bach JF, Carnaud C 1993 Evidence for a preferential V beta usage by the T cells which adoptively transfer diabetes in NOD mice. Eur J Immunol 23:727–733
223. Shizuru JA, Taylor-Edwards C, Livingstone A, Fathman CG 1991 Genetic dissection of T cell receptor V beta gene requirements for spontaneous murine diabetes. J Exp Med 174:633–638
224. Stein PH, Rees MA, Singer A 1992 Reconstitution of (BALB/c × B6)F1 normal mice with stem cells and thymus from nonobese diabetic mice results in autoimmune insulitis of the normal hosts' pancreases. Transplantation 53:1347–1352
225. Nakano K, Mordes JP, Handler ES, Greiner DL, Rossini AA 1988 Role of host immune system in BB/Wor rat. Predisposition to diabetes resides in bone marrow. Diabetes 37:520–525
226. Leiter EH, Serreze DV 1991 Autoimmune diabetes in the non-obese diabetic mouse: suppression of immune defects by bone marrow transplantation and implications for therapy. Clin Immunol Immunopathol 59:323–334
227. Gold DP, Bellgrau D 1991 Identification of a limited T-cell receptor beta chain variable region repertoire associated with diabetes in the BB rat. Proc Natl Acad Sci USA 88:9888–9891
228. Koevary SB, Blomberg M 1992 Prevention of diabetes in BB/Wor rats by intrathymic islet injection. J Clin Invest 89:512–516
229. Posselt AM, Barker CF, Friedman AL, Naji A 1992 Prevention of autoimmune diabetes in the BB rat by intrathymic islet transplantation at birth. Science 256:1321–1324
230. Kolb H, Zielasek J, Treichel U, Freytag G, Wrann M, Kiesel U 1986 Recombinant interleukin 2 enhances spontaneous insulin-dependent diabetes in BB rats. Eur J Immunol 16:209–212
231. Foulis AK, Farquharson MA, Hardman R 1987 Aberrant expression of class II major histocompatibility complex molecules by B cells and hyperexpression of class I major histocompatibility complex molecules by insulin containing islets in type 1 (insulin-dependent) diabetes mellitus. Diabetologia 30:333–343
232. Kay TW, Campbell IL, Oxbrow L, Harrison LC 1991 Overexpression of class I major histocompatibility complex accompanies insulitis in the non-obese diabetic mouse and is prevented by anti-interferon-gamma antibody. Diabetologia 34:779–785
233. Ono SJ, Issa-Chergui B, Colle E, Guttmann RD, Seemayer TA,

Fuks A 1988 IDDM in BB rats. Enhanced MHC class I heavy-chain gene expression in pancreatic islets. Diabetes 37:1411–1418
234. Hanafusa T, Fujino-Kurihara H, Miyazaki A, Yamada K, Nakajima H, Miyagawa J, Kono N, Tarui S 1987 Expression of class II major histocompatibility complex antigens on pancreatic B cells in the NOD mouse. Diabetologia 30:104–108
235. Signore A, Cooke A, Pozzilli P, Butcher G, Simpson E, Beverley PC 1987 Class-II and IL2 receptor positive cells in the pancreas of NOD mice. Diabetologia 30:902–905
236. Pujol-Borrell R, Todd I, Doshi M, Bottazzo GF, Sutton R, Gray D, Adolf GR, Feldmann M 1987 HLA class II induction in human islet cells by interferon-gamma plus tumour necrosis factor or lymphotoxin. Nature 326:304–306
237. Debray-Sachs M, Carnaud C, Boitard C, Cohen H, Gresser I, Bedossa P, Bach JF 1991 Prevention of diabetes in NOD mice treated with antibody to murine IFN gamma. J Autoimmun 4: 237–248
238. Campbell IL, Kay TW, Oxbrow L, Harrison LC 1991 Essential role for interferon-gamma and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. J Clin Invest 87: 739–742
239. Nicoletti F, Meroni PL, Landolfo S, Gariglio M, Guzzardi S, Barcellini W, Lunetta M, Mughini L, Zanussi C 1990 Prevention of diabetes in BB/Wor rats treated with monoclonal antibodies to interferon-gamma. Lancet 336:319
240. Shehadeh NN, Larosa F, Lafferty KJ 1993 Altered cytokine activity in adjuvant inhibition of autoimmune diabetes. J Autoimmun 6:291–300
241. Thivolet C, Bendelac A, Bedossa P, Bach JF, Carnaud C 1991 CD8+ T cell homing to the pancreas in the nonobese diabetic mouse is CD4+ T cell-dependent. J Immunol 146:85–88
242. Laufer TM, Von Herrath MG, Grusby MJ, Oldstone MB, Glimcher LH 1993 Autoimmune diabetes can be induced in transgenic major histocompatibility complex class II-deficient mice. J Exp Med 178:589–596
243. Bach JF, Boitard C, Yasunami R, Dardenne M 1990 Control of diabetes in NOD mice by suppressor cells. J Autoimmun 3:97–100
244. Dardenne M, Lepault F, Bendelac A, Bach JF 1989 Acceleration of the onset of diabetes in NOD mice by thymectomy at weaning. Eur J Immunol 19:889–895
245. Sempé P, Richard MF, Bach JF, Boitard C 1994 Evidence of CD4+ regulatory T cells in the nonobese diabetic male mouse. Diabetologia 37:337–343
246. Boitard C, Yasunami R, Dardenne M, Bach JF 1989 T cell-mediated inhibition of the transfer of autoimmune diabetes in NOD mice. J Exp Med 169:1669–1680
247. Pankewycz OG, Guan JX, Benedict JF 1992 A protective NOD islet-Infiltrating CD8+ T cell clone, I.S. 2.15, has *in vitro* immunosuppressive properties. Eur J Immunol 22:2017–2023
248. Pankewycz O, Strom TB, Rubin-Kelley VE 1991 Islet-infiltrating T cell clones from non-obese diabetic mice that promote or prevent accelerated onset diabetes. Eur J Immunol 21:873–879
249. Diaz-Gallo C, Moscovitch-Lopatin M, Strom TB, Kelley VR 1992 An anergic, islet-infiltrating T-cell clone that suppresses murine diabetes secretes a factor that blocks interleukin 2/interleukin 4-dependent proliferation. Proc Natl Acad Sci USA 89:8656–8660
250. Boitard C, Bendelac A, Richard MF, Carnaud C, Bach JF 1988 Prevention of diabetes in nonobese diabetic mice by anti-I-A monoclonal antibodies: transfer of protection by splenic T cells. Proc Natl Acad Sci USA 85:9719–9723
251. Kawamura T, Nagata M, Utsugi T, Yoon JW 1993 Prevention of autoimmune type I diabetes by CD4+ suppressor T cells in super-antigen-treated non-obese diabetic mice. J Immunol 151: 4362–4370
252. Formby B, Miller N, Peterson CM 1988 Adoptive immunotherapy of diabetes in autologous nonobese diabetic mice with lymphoid cells *ex vivo* exposed to cyclosporin plus interleukin 2. Diabetes 37:1305–1309
253. Greiner DL, Mordes JP, Handler ES, Angelillo M, Nakamura N, Rossini AA 1987 Depletion of RT6.1+ T lymphocytes induces diabetes in resistant biobreeding/Worcester (BB/W) rats. J Exp Med 166:461–475
254. Rossini AA, Faustman D, Woda BA, Like AA, Szymanski I, Mordes JP 1984 Lymphocyte transfusions prevent diabetes in the Bio-Breeding/Worcester rat. J Clin Invest 74:39–46
255. Mosmann TR, Coffman RL 1989 TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 7:145–173
256. Slattery RM, Miller JFAP, Heath WR, Charlton B 1994 Failure of a protective major histocompatibility complex class II molecule to delete autoreactive T cells in autoimmune diabetes. Proc Natl Acad Sci USA 90:10808–10810
257. Singer SM, Tisch R, Yang XD, McDevitt HO 1993 An Abdtransgene prevents diabetes in nonobese diabetic mice by inducing regulatory T cells. Proc Natl Acad Sci USA 90:9566–9570
258. Hanafusa T, Miyazaki A, Miyagawa J, Tamura S, Inada M, Yamada K, Shinji Y, Katsura H, Yamagata K, Itoh N, Asakawa H, Nakagawa C, Otsuka A, Kawata S, Kono N, Tarui S 1990 Examination of islets in the pancreas biopsy specimens from newly diagnosed type 1 (insulin-dependent) diabetic patients. Diabetologia 33:105–111
259. Kay TW, Campbell IL, Harrison LC 1991 Characterization of pancreatic T lymphocytes associated with beta cell destruction in the non-obese diabetic (NOD) mouse. J Autoimmun 4:263–276
260. Yang XD, Karin N, Tisch R, Steinman L, McDevitt HO 1993 Inhibition of insulitis and prevention of diabetes in nonobese diabetic mice by blocking L-selectin and very late antigen 4 adhesion receptors. Proc Natl Acad Sci USA 90:10494–10498
261. Strandell E, Elzirik DL, Sandler S 1990 Reversal of beta-cell suppression *in vitro* in pancreatic islets isolated from nonobese diabetic mice during the phase preceding insulin-dependent diabetes mellitus. J Clin Invest 85:1944–1950
262. Sempe P, Bedossa P, Richard MF, Villa MC, Bach JF, Boitard C 1991 Anti-alpha/beta T cell receptor monoclonal antibody provides an efficient therapy for autoimmune diabetes in nonobese diabetic (NOD) mice. Eur J Immunol 21:1163–1169
263. Shah SC, Malone JI, Simpson NE 1989 A randomized trial of intensive insulin therapy in newly diagnosed insulin-dependent diabetes mellitus. N Engl J Med 320:550–554
264. Bendtzen K, Mandrup-Poulsen T, Nerup J, Nielsen JH, Dinarello CA, Svenson M 1986 Cytotoxicity of human pI 7 interleukin-1 for pancreatic islets of Langerhans. Science 232:1545–1547
265. Pukel C, Baquerizo H, Rabinovitch A 1988 Destruction of rat islet cell monolayers by cytokines. Synergistic interactions of interferon-gamma, tumor necrosis factor, lymphotoxin, and interleukin 1. Diabetes 37:133–136
266. Pehuet-Figoni M, Ballot E, Bach JF, Chatenoud L 1992 The effects of interleukin-1 on pancreatic beta cell function *in vitro* depend on the glucose concentration. Eur Cytokine Netw 3: 443–450
267. Del Rey A, Besedovsky H 1989 Antidiabetic effects of interleukin 1. Proc Natl Acad Sci USA 86:5943–5947
268. Formby B, Jacobs C, Dubuc F, Shaw T 1992 Exogenous administration of IL-1 alpha inhibits active and adoptive transfer autoimmune diabetes in NOD mice. Autoimmunity 12:21–27
269. Jacob CO, Aiso S, Michie SA, McDevitt HO, Acha-Orbea H 1990 Prevention of diabetes in nonobese diabetic mice by tumor necrosis factor (TNF): similarities between TNF-alpha and interleukin 1. Proc Natl Acad Sci USA 87:968–972
270. Satoh J, Seino H, Abo T, Tanaka S, Shintani S, Ohta S, Tamura K, Sawai T, Nobunaga T, Oteki T, Kumagai K, Toyota T 1989 Recombinant human tumor necrosis factor alpha suppresses autoimmune diabetes in nonobese diabetic mice. J Clin Invest 84:1345–1348
271. Corbett JA, Mikhael A, Shimizu J, Frederick K, Misko TP, McDaniel ML, Kanagawa O, Unanue ER 1993 Nitric oxide production in islets from nonobese diabetic mice: aminoguanidine-sensitive and -resistant stages in the immunological diabetic process. Proc Natl Acad Sci USA 90:8992–8995
272. Bendelac A, Boitard C, Bedossa P, Bazin H, Bach JF, Carnaud C 1988 Adoptive T cell transfer of autoimmune nonobese diabetic mouse diabetes does not require recruitment of host B lymphocytes. J Immunol 141:2625–2628
273. Katz J, Benoist C, Mathis D 1993 Major histocompatibility complex class I molecules are required for the development of insulitis in non-obese diabetic mice. Eur J Immunol 23:3358–3360
274. Nagata M, Yokono K, Hayakawa M, Kawase Y, Hatamori N, Ogawa W, Yonezawa K, Shii K, Baba S 1989 Destruction of

pancreatic islet cells by cytotoxic T lymphocytes in nonobese diabetic mice. J Immunol 143:1155–1162
275. Young LH, Peterson LB, Wicker LS, Persechini PM, Young JD 1989 *In vivo* expression of perforin by CD8+ lymphocytes in autoimmune disease. Studies on spontaneous and adoptively transferred diabetes in nonobese diabetic mice. J Immunol 143:3994–3999
276. Boitard C, Chatenoud L, Debray-Sachs M 1982 *In vitro* inhibition of pancreatic B cell function by lymphocytes from diabetics with associated autoimmune diseases: a T cell phenomenon. J Immunol 129:2529–2531
277. Bradley BJ, Haskins K, La Rosa FG, Lafferty KJ 1992 CD8 T cells are not required for islet destruction induced by a CD4+ islet-specific T-cell clone. Diabetes 41:1603–1608
278. Maki T, Ichikawa T, Blanco R, Porter J 1992 Long-term abrogation of autoimmune diabetes in nonobese diabetic mice by immunotherapy with anti-lymphocyte serum. Proc Natl Acad Sci USA 89:3434–3438
279. Zavala AV, Fabiano de Bruno LE, Cardoso AI, Mota AH, Capucchio M, Poskus E, Fainboim L, Basabe JC 1992 Cellular and humoural autoimmunity markers in type 2 (non-insulin-dependent) diabetic patients with secondary drug failure. Diabetologia 35:1159–1164
280. Tuomi T, Groop LC, Zimmet PZ, Rowley MJ, Knowles W, MacKay IR 1993 Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. Diabetes 42:359–362
281. Groop L, Groop PH, Koskimies S 1986 Relationship between B-cell function and HLA antigens in patients with type 2 (non-insulin-dependent) diabetes. Diabetologia 29:757–760
282. Irvine WJ, Sawers JS, Feek CM, Prescott RJ, Duncan LJ 1979 The value of islet cell antibody in predicting secondary failure of oral hypoglycaemic agent therapy in diabetes mellitus. J Clin Lab Immunol 2:23–26
283. Tuomilehto-Wolf E, Tuomilehto J, Hitman GA, Nissinen A, Stengard J, Pekkanen J, Kivinen P, Kaarsalo E, Karvonen MJ 1993 Genetic susceptibility to non-insulin dependent diabetes mellitus and glucose intolerance are located in HLA region. Br Med J 307:155–159
284. Thai AC, Eisenbarth GS 1993 Natural history of IDDM. Diabetes Rev 1:1–14
285. Palmer JP 1993 Predicting IDDM. Use of humoral immune markers. Diabetes Rev 1:104–115
286. Tarn AC, Thomas JM, Dean BM, Ingram D, Schwarz G, Bottazzo GF, Gale EA 1988 Predicting insulin-dependent diabetes. Lancet 1:845–850
287. Riley WJ, MacLaren NK, Krischer J, Spillar RP, Silverstein JH, Schatz DA, Schwartz S, Malone J, Shah S, Vadheim C, Rotter JI 1990 A prospective study of the development of diabetes in relatives of patients with insulin-dependent diabetes. N Engl J Med 323:1167–1172
288. Barmeier H, McCulloch DK, Neifing JL, Warnock G, Rajotte RV, Palmer JP, Lernmark A 1991 Risk for developing type 1 (insulin-dependent) diabetes mellitus and the presence of islet 64K antibodies. Diabetologia 34:727–733
289. Gianani R, Pugliese A, Bonner-Weir S, Shiffrin AJ, Soeldner JS, Erlich H, Awdeh Z, Alper CA, Jackson RA, Eisenbarth GS 1992 Prognostically significant heterogeneity of cytoplasmic islet cell antibodies in relatives of patients with type I diabetes. Diabetes 41:1347–353
290. Lewis CM, Canafax DM, Sprafka JM, Barbosa JJ 1992 Double-blind randomized trial of nicotinamide on early-onset diabetes. Diabetes Care 15:121–123
291. Vague P, Picq R, Bernal M, Lassmann-Vague V, Vialettes B 1989 Effect of nicotinamide treatment on the residual insulin secretion in type 1 (insulin-dependent) diabetic patients. Diabetologia 32:316–321
292. Mendola G, Casamitjana R, Gomis R 1989 Effect of nicotinamide therapy upon B-cell function in newly diagnosed type 1 (insulin-dependent) diabetic patients. Diabetologia 32:160–162
293. Hutchings P, O'reilly L, Parish NM, Waldmann H, Cooke A 1992 The use of a non-depleting anti-CD4 monoclonal antibody to re-establish tolerance to beta cells in NOD mice. Eur J Immunol 22:1913–1918
294. Chatenoud L, Thervet E, Primo J, Bach JF 1993 Anti-CD3 antibody induces long-term remission of overt autoimmunity in non-obese diabetic mice. Proc Natl Acad Sci USA 91:123–127
295. Hurtenbach U, Ller E, Adorini L, Nagy ZA 1993 Prevention of autoimmune diabetes in non-obese diabetic mice by treatment with a class II major histocompatibility complex-blocking peptide. J Exp Med 177:1499–1504
296. Gerling IC, Serreze DV, Christianson SW, Leiter EH 1992 Intrathymic islet cell transplantation reduces beta-cell autoimmunity and prevents diabetes in NOD/Lt mice. Diabetes 41:1672–1676
297. Bougneres PF, Carel JC, Castano L, Boitard C, Gardin JP, Landais P, Hors J, Mihatsch MJ, Paillard M, Chaussain JL, Bach JF 1988 Factors associated with early remission of type 1 diabetes in children treated with cyclosporine. N Engl J Med 318:663–670
298. Silverstein J, MacLaren N, Riley W, Spillar R, Radjenovic D, Johnson S 1988 Immunosuppression with azathioprine and prednisone in recent-onset insulin-dependent diabetes mellitus. N Engl J Med 319:599–604
299. Cook JJ, Hudson I, Harrison LC, Dean B, Colman PG, Werther GA, Warne GL, Court JM 1989 Double-blind controlled trial of azathioprine in children with newly diagnosed type 1 diabetes. Diabetes 38:779–783
300. Bougneres PF, Landais P, Boisson C, Carel JC, Froment N, Boitard C, Chaussain JL, Bach JF 1990 Limited duration of remission of insulin dependency in children with recent overt type 1 diabetes treated with low-dose cyclosporin. Diabetes 39:1264–1272
301. Burcelin RG, Eddouks M, Beylot M, Normand S, Boitard C, Peutren G, Landais P, Riou JF, Girard JR, Bach JF, Assan RI 1993 Hypersensitivity to insulin during remissions in cyclosporin-treated IDDM patients. Diabetes Care 16:881–888
302. Hramiak IM, Dupre J, Finegood DT 1993 Determinants of clinical remission in recent-onset IDDM. Diabetes Care 16:125–132
303. Ogawa M, Maruyama T, Hasegawa T, Kanaya T, Kobayashi F, Tochino Y, Uda H 1985 The inhibitory effect of neonatal thymectomy on the incidence of insulitis in non-obese diabetic (NOD) mice. Biomed Res 6:103–105
304. Mori Y, Suko M, Okudaira H, Matsuba I, Tsuruoka A, Sasaki A, Yokoyama H, Tanase T, Shida T, Nishimura M, Terada E, Ikeda Y 1986 Preventive effects of cyclosporin on diabetes in NOD mice. Diabetologia 29:244–247
305. Miyagawa J, Yamamoto K, Hanafusa T, Itoh N, Nakagawa C, Otsuka A, Katsura H, Yamagata K, Miyazaki A, Kono N, Tarui S 1990 Preventive effect of a new immunosuppressant FK-506 on insulitis and diabetes in non-obese diabetic mice. Diabetologia 33:503–505
306. Strasser S, Cearns-Spielman J, Carroll P, Alejandro R 1992 Effect of FK 506 on cyclophosphamide-induced diabetes in NOD mice. Diabetes Nutr Metab 5:61–63
307. Nicoletti F, Borghi MO, Meroni PL, Barcellini W, Fain C, Di Marco R, Menta R, Schorlemmer HU, Bruno G, Magros G, Grasso S 1992 Prevention of cyclophosphamide-induced diabetes in the NOD/WEHI mouse with deoxyspergualin. Clin Exp Immunol 91:232–236
308. Baeder WL, Sredy J, Sehgal SN, Chang JY, Adams LM 1992 Rapamycin prevents the onset of insulin-dependent diabetes mellitus (IDDM) in NOD mice. Clin Exp Immunol 89:174–178
309. Hayward AR, Schriber M, Cooke A, Waldmann H 1993 Prevention of diabetes but not insulitis in NOD mice injected with antibody to CD4. J Autoimmun 6:301–310
310. Wang Y, Hao L, Gill RG, Lafferty KJ 1987 Autoimmune diabetes in NOD mouse is L3T4 T-lymphocyte dependent. Diabetes 36:535–538
311. Koike T, Itoh Y, Ishii T, Ito I, Takabayashi K, Maruyama N, Tomioka H, Yoshida S 1987 Preventive effect of monoclonal anti-L3T4 antibody on development of diabetes in NOD mice. Diabetes 36:539–541
312. Shizuru JA, Taylor-Edwards C, Banks BA, Gregory AK, Fathman CG 1988 Immunotherapy of the nonobese diabetic mouse: treatment with an antibody to T-helper lymphocytes. Science 240:659–662
313. Hutchings PR, Simpson E, O'reilly LA, Lund T, Waldmann H, Cooke A 1990 The involvement of Ly2+ T cells in beta cell destruction. J Autoimmun 3 [Suppl 1]:101–109
314. Taki T, Nagata M, Ogawa W, Hatamori N, Hayakawa M, Hari J, Shii K, Baba S, Yokono K 1991 Prevention of cyclophospha-

mide-induced and spontaneous diabetes in NOD/Shi/Kbe mice by anti-MHC class I Kd monoclonal antibody. Diabetes 40:1203–1209
315. Kelley VE, Gaulton GN, Hattori M, Ikegami H, Eisenbarth G, Strom TB 1988 Anti-interleukin 2 receptor antibody suppresses murine diabetic insulitis and lupus nephritis. J Immunol 140: 59–61
316. Pacheco-Silva A, Bastos MG, Muggia RA, Pankewycz O, Nichols J, Murphy JR, Strom TB, Rubin-Kelley VE 1992 Interleukin 2 receptor targeted fusion toxin (DAB486-IL-2) treatment blocks diabetogenic autoimmunity in non-obese diabetic mice. Eur J Immunol 22:697–702
317. Rabinovitch A, Suarez WL, Power RF 1992 Combination therapy with an antioxidant and a corticosteroid prevents autoimmune diabetes in NOD mice. Life Sci 51:1937–1943
318. Mathieu C, Laureys J, Sobis H, Vandeputte M, Waer M, Bouillon R 1992 1,25-Dihydroxyvitamin D3 prevents insulitis in NOD mice. Diabetes 41:1491–1495
319. Wilberz S, Herberg L, Renold AE 1988 Ganglioside *in vivo* reduce diabetes incidence in non-obese diabetic mice. Diabetologia 31:855–857
320. Pearce RB, Peterson CM 1991 Studies of concanavalin A in nonobese diabetic mice. I. Prevention of insulin-dependent diabetes. J Pharmacol Exp Ther 258:710–715
321. Yamada K, Nonaka K, Hanafusa T, Miyazaki A, Toyoshima H, Tarui S 1982 Preventive and therapeutic effects of large-dose nicotinamide injections on diabetes associated with insulitis. An observation in nonobese diabetic (NOD) mice. Diabetes 31: 749–753
322. Foragren S, Andersson A, Hillorn V, Soderstrom A, Holmberg D 1991 Immunoglobulin-mediated prevention of autoimmune diabetes in the non-obese diabetic (NOD) mouse. Scand J Immunol 34:445–451
323. Charlton B, Baelj A, Mandel TE 1988 Administration of silica particles or anti-Lyt2 antibody prevents beta-cell destruction in NOD mice given cyclophosphamide. Diabetes 37:930–935
324. Like AA, Kislauskis E, Williams RR, Rossini AA 1982 Neonatal thymectomy prevents spontaneous diabetes mellitus in the BB/W rat. Science 216:644–646
325. Laupacis A, Stiller CR, Gardell C, Keown P, Dupre J, Wallace AC, Thibert P 1983 Cyclosporin prevents diabetes in BB Wistar rats. Lancet 1:10–12
326. Jaworski MA, Honore L, Jewell LD, Mehta JG, McGuire-Clark P, Schouls JJ, Yap WY 1986 Cyclosporin prophylaxis induces long-term prevention of diabetes, and inhibits lymphocytic infiltration in multiple target tissues in the high-risk BB rat. Diabetes Res 3:1–6
327. Like AA, Dirodi V, Thomas S, Guberski DL, Rossini AA 1984 Prevention of diabetes mellitus in the BB/W rat with Cyclosporin-A. Am J Pathol 117:92–97
328. Like AA, Rossini AA, Guberski DL, Appel MC, Williams RM 1979 Spontaneous diabetes mellitus: reversal and prevention in the BB/W rat with antiserum to rat lymphocytes. Science 206:1421–1423
329. Boitard C, Michie S, Serrurier P, Butcher GW, Larkins AP, McDevitt HO 1985 *In vivo* prevention of thyroid and pancreatic autoimmunity in the BB rat by antibody to class II major histocompatibility complex gene products. Proc Natl Acad Sci USA 82:6627–6631
330. Rossini AA, Slavin S, Woda BA, Geisberg M, Like AA, Mordes JP 1984 Total lymphoid irradiation prevents diabetes mellitus in the Bio-Breeding/Worcester (BB/W) rat. Diabetes 33:543–547
331. Sato J, Seino H, Shintani S, Tanaka S, Ohteki T, Masuda T, Nobunaga T, Toyota T 1990 Inhibition of type 1 diabetes in BB rats with recombinant human tumor necrosis factor-alpha. J Immunol 145:1395–1399
332. Vialettes B, Picq R, Du Rostu M, Charbonnel B, Rodier M, Mirouze J, Vexiau P, Passa P, Fehuet M, Elgrably F, Vague P 1990 A preliminary multicentre study of the treatment of recently diagnosed type 1 diabetes by combination nicotinamide-cyclosporin therapy. Diabetic Med 7:731–735
333. Atkison PR, Mahon JL, Dupre J, Stiller CR, Jenner MR, Paul TL, Momah CI 1990 Interaction of bromocriptine and cyclosporine in insulin dependent diabetes mellitus: results from the Canadian open study. J Autoimmun 3:793–799
334. Carroll PB, Tzakis AG, Ricordi C, Rilo HR, Abu-El-Magd K, Murase N, Zeng YJ, Alejandro R, Mintz D, Starzl TE 1991 The use of FK 506 in new-onset type 1 diabetes in man. Transplant Proc 23:3351–3353
335. Secchi A, Pastore MR, Sergi A, Pontiroli AE, Pozza G 1990 Prednisone administration in recent onset type I diabetes. J Autoimmun 3:593–600
336. Moncada E, Subira ML, Oleaga A, Goni F, Sanchez-Ibarrola A, Monreal M, Sevilla M, Goni MJ, Yoldi A, Teran D, Llorente I 1990 Insulin requirements and residual beta-cell function 12 months after concluding immunotherapy in type I diabetic patients treated with combined azathioprine and thymostimulin administration for one year. J Autoimmun 3:625–638
337. Panto F, Giordano C, Amato MP, Pugliese A, Donatelli M, D'Acquisto G, Galluzzo A 1990 The influence of high dose intravenous immunoglobulins on immunological and metabolic pattern in newly diagnosed type I diabetic patients. J Autoimmun 3:587–592
338. Krug J, Verlohren HJ, Bierwolf B, Lampeter E, Borte M, Nietzschmann U, Haustein B, Lohmann D 1990 Lymphocyte transfusion in recent onset type I diabetes mellitus—a one-year follow-up of cell-mediated anti-islet cytotoxicity and C-peptide secretion. J Autoimmun 3:601–609
339. Dempe A, Baaske W, Von Baehr R, Kuttner S, Neubert G, Neumeister K 1988 Remission of the newly diagnosed type 1 diabetes by radiation of the pancreas. Exp Clin Endocrinol 92: 123–125
340. Giordano C, Pantoo F, Amato MP, Sapienza N, Pugliese A, Galluzzo A 1990 Early administration of an immunomodulator and induction of remission in insulin-dependent diabetes mellitus. J Autoimmun 3:611–617

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Clinical significance of IgG Fc receptors and FcγR-directed immunotherapies

Yashwant M. Deo, Robert F. Graziano, Roland Repp and Jan G.J. van de Winkel

Fc receptors for IgG (FcγRs) can trigger the inflammatory, cytotoxic and hypersensitivity functions of immune effector cells. Activation or deactivation of effector cells via FcγRs can be exploited to develop novel therapies for cancer, infectious diseases and autoimmune disorders. Initial results of clinical trials for several FcγR-directed immunotherapies show the potential promise of this approach.

The IgG Fc receptors (FcγRs) are expressed primarily on immune effector cells, and link cellular and humoral immunity by serving as a bridge between antibody specificity and effector cell function. In this fashion, FcγRs act as trigger molecules for inflammatory, cytolytic, allergic (hypersensitivity), endocytic and phagocytic activities of immune effector cells. Moreover, since many FcγR-bearing cells are also antigen-presenting cells (APCs; e.g. macrophages, dendritic cells), FcγR-mediated internalization via phagocytosis may also lead to antigen presentation and amplification of the immune response. These functions of FcγRs are linked to activation and regulation of immune defense in various disease conditions. The position of FcγRs as a gateway both to cellular and humoral aspects of the immune cascade makes them potentially attractive candidates for directed immunotherapy. This review focuses on the clinical significance of FcγRs and developments in FcγR-directed therapies for cancer, infectious diseases and autoimmune disorders.

FcγR structure and function

There are three classes of FcγR: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). These classes comprise nine membrane-associated and three soluble FcγR molecules, encoded by eight genes (Fig. 1a). FcγRs are expressed by most hematopoietic cells, and their expression can be enhanced by certain inflammatory cytokines such as interferon γ (IFN-γ) and granulocyte colony-stimulating factor (G-CSF) (Table 1)[1,2]. With the exception of the glycosylphosphatidylinositol (GPI)-linked FcγRIIIb, all FcγRs are transmembrane molecules belonging to the family of multichain immune recognition receptors (MIRRs), which also includes the B-cell receptor (BCR) and T-cell receptor (TCR). FcγRIa is a high-affinity receptor and contains three Ig-like domains in its extracellular region, instead of two as in all other FcγRs. FcγRII and FcγRIII represent low-affinity receptors. Most FcγRs exist as hetero-oligomeric complexes with a ligand-binding α-chain and a signaling component comprising γ-, β- or ζ-chains (Fig. 1a, Table 1). Each signaling chain bears a unique ~26 amino acid immunoreceptor tyrosine-based activation motif (ITAM) involved in activatory functions. A similar, albeit noncanonical, ITAM is located in the cytoplasmic region of FcγRIIa and appears to be critical for cell activation by this receptor[3]. Recently, FcγRIIa has been shown to be capable of interacting with the FcR γ-chain, which modulates its signaling behavior[4–6]. The FcγRIIb members contain a unique 13 amino acid immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domain, and this is important in immune-inhibitory functions. Conserved tyrosine and leucine residues within these signaling motifs play a central role in FcγR signal transduction[3].

Additional FcγR heterogeneity is introduced by polymorphisms (Fig. 1b). The myeloid FcγRIIa (CD32) differs by a single amino acid within the second Ig-like domain, either an arginine or histidine at position 131 (FcγRIIa-R131 or FcγRIIa-H131)[7]. The neutrophil FcγRIIIb-NA1 and -NA2 allotypes differ by five nucleotides, which result in an increased number of glycosylation sites in FcγRIIIb-NA2 (six versus four)[8]. In addition, amino acid variation at position 48 distinguishes three allotypes of FcγRIIIa (*Ref. 8*). Furthermore, amino acid variation at position 158 of FcγRIIIa results in a polymorphism with functional consequences (H.R. Koene *et al.*, unpublished).

Although the extracellular domains of various FcγRs do not exhibit exclusive specificity for ligands (Table 1), individual FcγRs trigger characteristic biological responses determined by both the nature of the effector cell and the transmembrane and cytoplasmic regions of the receptor[1,2]. Furthermore, the transmembrane domains of MIRRs may functionally interact. For example: on neutrophils, crosslinking of FcγRIIb enhances FcγRIIa-mediated phagocytosis[9]; on B cells, co-crosslinking of FcγRIIb and the BCR results in down-modulation of antibody secretion; and, on neutrophils, complement receptor 3 (CR3; CD11b/CD18) acts as a signaling partner for GPI-linked FcγRIIIb (Refs 5, 10, 11). The first step in FcγR activation is receptor crosslinking, with as few as two crosslinked receptors activating the signaling cascade (Fig. 2). Crosslinking at the FcγR-ligand-binding domain, as well as outside this domain (via anti-receptor monoclonal antibodies (mAbs)), triggers FcγR function[1,12]. The second step involves phosphorylation of tyrosine residues

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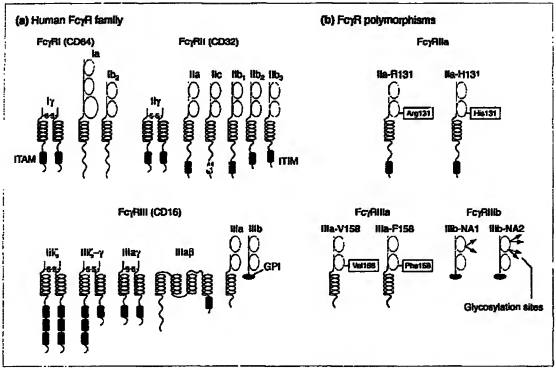


Fig. 1. (a) *The human FcγR family. The ligand-binding α-chains of all receptors contain extracellular regions comprising disulfide-bonded immunoglobulin (Ig)-like domains. FcγRI has three Ig-like domains, the others have two Ig-like domains. FcγRIa, FcγRIIa and FcγRIIIa exist as oligomeric complexes with associated FcR γ-, β- or ζ-chains, which contain immunoreceptor tyrosine-based activation motifs (ITAMs) indicated by the plus sign. FcγRIIb molecules contain an inhibitory motif (ITIM), indicated by the minus sign. All three classes contain soluble molecules not shown in this diagram.* (b) *FcγR polymorphisms. Two allotypic forms of human FcγRIIa have been distinguished by the presence of either arginine (FcγRIIa-R131) or histidine (FcγRIIa-H131) at position 131. The two allotypes of FcγRIIIa contain either valine or phenylalanine at position 158. The FcγRIIIb-NA1 and -NA2 allotypes differ by five nucleotides, which results in differential glycosylation (indicated by the arrow heads). Abbreviation: GPI, glycosylphosphatidylinositol.*

within the ITAM of FcγRs by *src*-family protein tyrosine kinases (PTKs). This is followed by association and activation of *syk*-family PTKs with the phosphorylated ITAM. The subsequent events are not clearly delineated but appear to involve several distinct signaling components leading to different biological responses[1].

FcγR-expressing cells activated via these signaling cascades are able to lyse or phagocytose IgG-opsonized prokaryote or tumor cells, as well as clear immune complexes (ICs), promote antigen presentation and induce inflammation. The FcγR-dependent phagocytic and cytolytic [antibody-dependent cellular cytotoxicity (ADCC)] activities are well documented. These activities play a key role in immune defense against infectious diseases, and probably in immune surveillance against malignant cell growth. *In vitro*, targeting antigens to FcγRs on macrophages and dendritic cells significantly facilitates antigen presentation[13]. Similar data have been obtained in a human FcγRI (huFcγRD transgenic mouse model in which the transgenic animals induced a much greater humoral response to FcγRI-directed antigens than the nontransgenic littermates, supporting a role for huFcγRI in antigen presentation[14]. In the same model, a role for FcγRI in inflammatory processes was suggested by a dramatic increase in phagocyte expression of huFcγRI in mice with inflammatory lesions. In contrast to these immune defense functions, activation of FcγR by autoantibodies or defects in FcγR functions are implicated in several autoimmune disorders. Recently, the significance of FcγRs in type II and III hypersensitivity reactions has been firmly established by defective anaphylactic and inflammatory responses observed in mice deficient in the FcR γ-chain or in FcγRIII (Refs 15–17). Thus, the pleiotropic biological responses induced via FcγRs play a significant role in various diseases. Therefore, therapies that harness these cytotoxic and immune activation functions of FcγRs, or downmodulate FcγR activity, are currently being developed.

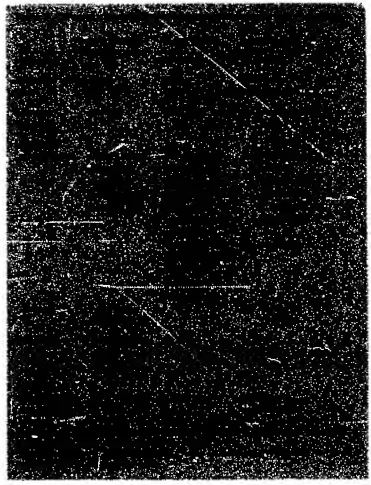
FcγRs and cancer

Destruction of tumor cells by FcγR-expressing effectors via ADCC and phagocytosis has been well established. Tumor-specific antibodies and bispecific molecules (BSMs) directed to FcγR-expressing effector cells represent two approaches developed to harness FcγR activities for cancer therapy. FcγR-directed tumor vaccines are also

being developed, since antigens directed to FcγRs on APCs induce strong antigen-specific immune activation[13,14]

Role of FcγRs in antibody therapy

Tumor-specific mAbs can mediate destruction of tumor cells by phagocytosis or ADCC induced via binding to FcγRs. *In vitro* studies have shown mAb-mediated ADCC of a broad spectrum of tumor cell lines, derived both from solid tumors and hemato-lymphatic tumors, by FcγR-expressing monocytes, macrophages, eosinophils, neutrophils and natural killer (NK) cells[9,10]. Involvement of FcγRs in mAb-mediated cytotoxicity is supported by the following observations: (1) crosslinking FcγRs triggers cytotoxicity of specific immune effector cells; (2) serum IgG, which can compete with tumor-specific mAbs of certain isotypes for binding to FcγRs, inhibits mAb-mediated ADCC of tumor cells; (3) mAb-mediated tumoricidal activity of specific effector cells can be induced or enhanced by cytokines that upregulate FcγR expression[9]; (4) antitumor activity of different isotypes correlates with the ability of an isotype to engage FcγRs on cytotoxic effector cells; and (5) with a few exceptions, $F(ab')_2$ fragments of tumor-specific mAbs are ineffective in tumor cell killing.



In vivo studies in mouse models and clinical trials further support the *in vitro* observations. First, tumor-specific mAbs have been found to be equally effective in eradicating tumors in mice deficient in complement component C5 as in control mice, which thereby excludes complement-mediated tumor cell lysis in this model[11]. Furthermore, the capacity of antibodies to elicit tumor regression has been shown in certain cases to depend on FcγR-expressing effector cells[12]. Indeed, the rate of tumor rejection correlates with the density of FcγR-expressing effector cell infiltration at the tumor site following antibody therapy, and depletion of FcR^+ effector cells was found to abrogate mAb efficacy[12,13]. In addition, comparison of antibodies with the same tumor specificity but different isotypes shows a correlation between the capacity of an antibody to induce ADCC *in vitro* and its efficacy *in vivo* in mouse models[14]. In a clinical trial comparing isotype switch variants of CAMPATH antibody (specific for CD w52), the strongest depletion of malignant cells was observed with the antibody isotype that most effectively induced ADCC *in vitro*[15]. Adjuvant therapy with a murine IgG2a (a potent mediator of ADCC) tumor-specific mAb (anti-17-1A) reduced the overall death rate by >30% in colorectal cancer patients[16]. Human IgG1 has the broadest spectrum reactivity with human FcγRs (Table 1) and is, therefore, regarded as optimal for effector cell recruitment. In accordance, a humanized IgG1 anti-HER-2/*neu* antibody and a chimeric IgG1 anti-CD20 antibody have shown very encouraging clinical responses, emphasizing the importance of the human Fc region[18,19]. These studies indicate that the cytotoxic activity of FcγR-expressing effector cells may play an important role in the antitumor effects of tumor-reactive mAbs.

BSMs

In order to improve effector cell recruitment and FcγR activation at tumor sites, BSMs that have one arm specific for tumor cells and the other specific for FcγRs on immune effector cells have been developed[12]. These BSMs offer several advantages over conventional mAbs as detailed in Box 1.

FcγRI and FcγRIII are of particular interest for BSM targeting. FcγRI is expressed solely on cytotoxic effector cells and is always capable of triggering cytotoxic activity. Since it is typically saturated

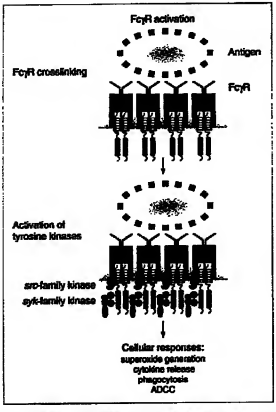


Fig. 2. *Schematic representation of effector cell activation through FcγRs. The crucial first step is crosslinking of FcγRs, and this is promoted by simultaneous binding of several antigen–IgG immune complexes to the extracellular region of FcγR α-chains. This results in the association and activation of src-family PTKs, inducing tyrosine phosphorylation (P) of the FcγR ITAM. This phosphorylation results in binding and activation of syk-family PTKs, followed by a cascade of events culminating in physiological responses. The exact point(s) of interaction between the PTKs and FcγRs has not been well established. Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ITAM, immunoreceptor tyrosine-based activation motif; PTK, protein tyrosine kinase.*

with serum IgG under normal physiological conditions, it can be most effectively triggered to induce ADCC, phagocytosis and other effector functions by BSMs that can bind outside the Fc ligand-binding domain. Several sc_{Fv} BSMs have been developed, with one arm specific for FcγRI and the other arm specific for a tumor marker such as CD15, HER-2/*neu*, epidermal growth factor receptor (EGFR)[17], disialoganglioside (GD2), HLA-DR (Ref. 27), CD19, CD37, or gastrin-releasing peptide (GRP) receptor (reviewed in Ref. 12). These BSMs readily direct monocytes, macrophages and IFN-γ- or G-CSF-activated polymorphonuclear leukocytes (PMNs) to tumor cells, and have proven to be highly effective in mediating ADCC and phagocytosis of tumor targets. FcγRIII is an important Fc receptor triggering ADCC by NK cells and it is also functional on macrophages (Table 1). BSMs specific for FcγRIII and tumor antigens

Box 1. Advantages of bispecific molecules (BSMs)

- BSMs are relatively small (50–100 kDa) and may penetrate tumors better than monoclonal antibodies (mAbs) (150–1000 kDa)
- BSMs can efficiently mediate effector cell (macrophage, neutrophil or natural killer (NK) cell)-dependent lysis of monolayers or spheroids of tumor cells
- BSMs can be constructed with or without an Fc region to retain or eliminate complement-activating capacity
- BSMs can be designed selectively to trigger FcγRs expressed solely on cytotoxic effector cells (e.g. FcγRI) to avoid triggering of non-cytotoxic cells (e.g. platelets or B cells that express FcγRII)
- BSMs can be constructed to bind to an epitope on FcγRs outside the FcR-binding domain to circumvent competition by serum IgG for FcγR binding and to maintain antitumor activity in the presence of serum IgG
- BSMs can be designed to target specifically FcγRs on phagocytic cells, which function both as cytotoxic effectors and antigen-presenting cells, to promote tumor destruction and tumor-specific immunity
- BSMs can be designed to target to tumor cells in order to engage, if necessary, those effector cells may be 'armed' with BSMs, conferring on them specific antitumor activity

such as HER-2/*neu*, CD30, CA19-9, CD33 and high-molecular-weight melanoma antigen have shown effective killing of tumor cells *in vitro*. Efficacy of BSMs *in vivo* has been demonstrated in severe combined immunodeficiency (SCID) mice xenografted with human tumors. BSMs in combination with human effector cells induced long-term survival[28] and complete regression of established tumors[29].

Three BSMs directed to FcγRI and two directed to FcγRIII are currently being tested in clinical trials, either alone or in combination with cytokines that may enhance their efficacy (Table 2). Several phase I/II studies are under way with two BSMs (MDX-210 and MDX-447) comprising chemically linked F(ab') fragments of FcγRI- and HER-2/*neu*- or EGFR-specific antibodies, in late-stage cancer patients with various HER-2/*neu*$^+$ or EGFR$^+$ malignancies. Single and multiple doses (up to 25 mg m^{-2}) of BSMs are tolerated well, and induce immunological and biological responses[30,31]. After infusion, BSMs bind rapidly to FcγRI-expressing effector cells, and trigger both a transient disappearance of these cells from the circulation and a significant rise in serum levels of the inflammatory cytokines, tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and G-CSF. BSM-coated effector cells infiltrate tumors, resulting in tumor inflammation, tumor regression, a decrease in levels of tumor antigen in circulation, and improvement in symptomatic relief[30,31]. In some instances, up to 20-fold increases in serum levels of human antitumor antibodies (IgM and IgG) were observed, indicating that FcγRI-directed BSMs promote antigen presentation and induction of antitumor immune responses *in vivo* (P. Guyre *et al.*, unpublished). In another trial, a BSM comprising a mAb to CD15 linked with an FcγRI mAb was tested in

four patients, one of which showed a transient decrease in leukemic cells[32]. A BSM specific for FcγRIII and CD30 has been tested in patients with Hodgkin's disease and shown to be tolerated well and able to elicit a clinical response in some patients (F. Hartmann *et al.*, unpublished). A BSM (ZB1) specific for FcγRIII and HER-2/*neu*, and comprising a hetero-antibody containing the murine IgG1 Fc region, has been tested in HER-2/*neu*$^+$ patients[33]. Multiple doses of ZB1 induced elevated serum levels of TNF-α, IL-6, IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF) and IFN-γ, as well as minor clinical responses. Notably, several patients exhibited significant increases in human anti-HER-2/*neu* antibodies of both IgM and IgG isotypes, indicating that ZB1 treatment induced specific antitumor immune cascades (J. Graslow *et al.*, unpublished). These encouraging results from clinical trials point to the potential promise of FcγR-directed BSMs in cancer therapy.

FcγRs and infectious diseases

FcγRs are of crucial importance in directing the uptake and destruction of viruses, bacteria and a variety of infectious parasites, and are involved in antibody-dependent killing of infected cells expressing viral antigens[1,12]. FcγRIIIa-expressing NK cells isolated from human immunodeficiency virus (HIV)-seropositive individuals have been shown to be coated with anti-HIV antibodies and readily mediate lysis of HIV-infected or gp120-coated target cells *in vitro*. Furthermore, this ADCC activity correlates inversely with disease progression[34]. The importance of appropriate detection of IgG-opsonized microorganisms by FcγRs on phagocytes is further emphasized by susceptibility of individuals expressing the FcγRIIa-R131 allotype to infections by encapsulated bacteria. The FcγIIa-H131 allotype (as opposed to FcγRIIa-R131) is identified as the only FcγR capable of binding human IgG2 (Ref. 6), an important isotype in immune defense against encapsulated bacteria. Neutrophils from individuals expressing the FcγRIIa-R131 allotype inefficiently phagocytose human IgG2-coated bacteria[35], rendering these individuals more susceptible to infection. Allotypic forms of FcγRIIIb (NA1 versus NA2) have also demonstrated differences in the binding and phagocytosis of IgG1- and IgG3-coated particles[36], which may have clinical relevance with regard to susceptibility to infectious disease.

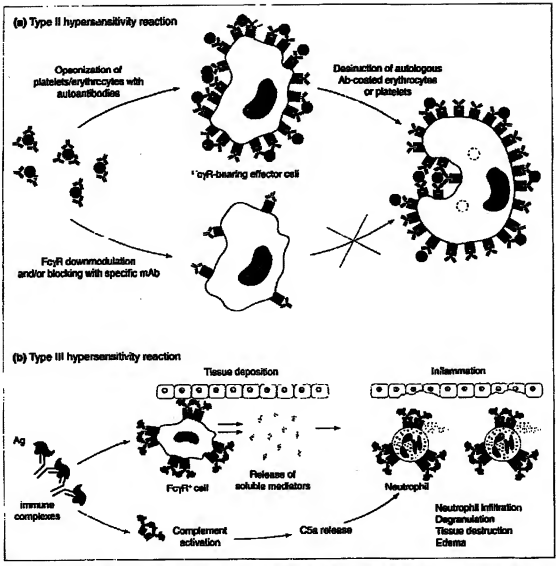


Fig. 3. *Role of FcγRs in type II and III hypersensitivity. (a) Type II hypersensitivity is induced when Abs bind to Ag(s) on autologous cells such as platelets or erythrocytes. Opsonized cells are then destroyed upon encountering FcγR-bearing effector cells, resulting in autoimmune disorders such as ITP (when the target cell is a platelet) or AIHA (when the target cell is an erythrocyte). ITP has been treated with molecules that downmodulate or block FcγRs to prevent platelet destruction. (b) Type III hypersensitivity is induced when circulating immune complexes that have not been properly cleared by FcγR-bearing cells of the mononuclear phagocyte system deposit at tissue sites. Ag–Ab complexes encounter FcγR-bearing cells and trigger the release of soluble mediators. These mediators initiate a series of events, including tissue edema and infiltration of neutrophils. The neutrophils mediate tissue destruction upon engagement of FcγRs by immune complexes or anti-FcγR autoantibodies. Abbreviations: Ab, antibody; Ag, antigen; AIHA, autoimmune hemolytic anemia; ITP, idiopathic thrombocytopenia purpura; mAb, monoclonal antibody.*

FcγRs are also important for immune defense to intracellular pathogens such as *Toxoplasma gondii*. Antibodies specific for *T. gondii* focus the organism to the effector cell by binding to FcγRs, thereby leading to destruction of the pathogen. BSMs that focus *T. gondii* to the surface of myeloid effectors (monocytes and neutrophils) mediate destruction of the pathogen regardless of the surface antigen on the effector cell to which they are directed[29]. In contrast to phagocytes, NK cells destroy *T. gondii* only upon targeting to FcγRIII, and not other cell-surface markers, identifying FcγRIII on NK cells as the primary trigger molecule for *T. gondii* destruction[29]. BSMs are

now being developed for a variety of microorganisms, including fungi and antibiotic-resistant bacterial strains, to target these pathogens specifically to FcγR-expressing cytotoxic effector cells.

Antibody-dependent enhancement

Another interaction between pathogens and FcγRs is constituted by the phenomenon of antibody-dependent enhancement (ADE) of infection by certain viruses. Sufficient level of opsonization by virus-specific antibodies leads to FcγR crosslinking, internalization and degradation of opsonized virus particles. However, in some instances, suboptimal levels of virus-specific antibodies have been found to promote infection of FcγR$^+$ cells by flavi viruses, alpha viruses, rhabdoviruses and retroviruses[1]. Also, *in vitro*, BSMs that target dengue virus to FcγRI or FcγRIIa, or to non-FcγR surface antigens, can mediate ADE by focusing virus to the cell surface[38]. On the other hand, BSMs that direct HIV to FcγRI, FcγRII or FcγRIII on monocyte-derived macrophages markedly reduced virus production with no evidence of ADE (*Ref.* 39). However, a BSM targeting HIV to a non-FcγR surface antigen (CD33) was ineffective and even led to ADE of macrophages. Thus, the evidence for FcγR-mediated ADE is not conclusive.

Another gp41- and FcγRI-specific BsM (MDX-240) has been shown to decrease virus production significantly, as well as diminish formation of HIV proviral DNA in macrophages. In a phase I/II clinical trial, up to six 10 μg m^{-2} doses of MDX-240 were tolerated well, and induced a transient increase in CD4$^+$ T cells in some patients, although none of the treated patients showed evidence of ADE (J.L. Pasquali *et al.*, unpublished). These studies establish the pleiotropic role of FcγRs in infectious disease processes and identify FcγR-directed BSMs as a potential therapeutic approach.

FcRs and autoimmune disorders

FcγRs have been shown to play a significant role in autoimmune disorders, either by mediating destruction of normal cells opsonized with autoantibodies or, conversely, by failing to clear ICs adequately. For example, inability of FcγR-bearing cells to remove soluble ICs has been proposed to enhance autoimmune conditions such as systemic lupus erythematosus (SLE), where IC deposition in tissues triggers inflammation and tissue destruction, a characteristic type III hypersensitivity reaction (Fig. 2a). On the other hand, engagement of functional FcRs on effector cells of the mononuclear phagocyte system triggers the destruction of autologous erythrocytes or platelets in the presence of autoantibodies directed to these cells. This may result in autoimmune hemolytic anemia (AIHA) or idiopathic thrombocytopenia purpura (ITP), both of which are autoimmune disorders characteristic of the type II hypersensitivity class of inflammation (Fig. 3). These observations suggest that FcγR-directed therapies could be developed to treat autoimmune disorders mediated by either type II or III hypersensitivity reactions.

SLE patients characteristically make autoantibodies specific for double-stranded (ds)DNA and other nuclear factors. The ICs formed by these antibodies deposit in the kidney and cause renal dysfunction because of insufficient clearance by phagocytes. FcRs in these patients may be downregulated or uncoupled from the signal transduction cascade[9,41]. FcRs may also play a role in the inflammation and tissue destruction observed in SLE patients[15]. Tissue-deposited ICs crosslink FcRs on infiltrating immune effector cells (neutrophils and macrophages), causing the release of inflammatory cytokines, proteolytic enzymes and other toxic molecules (Fig. 3)[42]. The presence of anti-FcγR autoantibodies in the sera of patients with autoimmune diseases has been proposed to explain the role of impaired FcγR function[43]. Anti-FcγRI, -II or -III autoantibodies have been purified from the sera of patients with SLE, Sjögren's syndrome, rheumatoid arthritis, Raynaud's disease and progressive systemic sclerosis. These may not only affect IC clearance, but can also crosslink FcγRs and trigger release of proinflammatory molecules[43]. Soluble FcγRs have been demonstrated to inhibit the Arthus reaction, implicating a role for FcγRs in type III hypersensitivity reaction[44]. Recent studies, demonstrating drastically reduced Arthus reaction in FcR γ-chain-deficient and FcγRIII-deficient mouse models, have established that FcγRs play an important role in type III hypersensitivity reactions[13,17].

FcγR polymorphisms also seem relevant in autoimmune disease. A marked skewing of FcγRIIa alleleypes that interact differently with human IgG2 and IgG3 isotypes has been observed in Caucasian SLE patients with lupus nephritis[45], and in African–American SLE patients, both with and without lupus nephritis[46]. Several clinical parameters were found more frequently in FcγRIIa-R/R131 than in FcγRIIa-H/H131 homozygous patients, including high levels of anti-dsDNA and anti-Sm autoantibodies, as well as increased incidence of AIHA (R. Repp and J.G.J. van de Winkel, unpublished). Furthermore, this polymorphism seems important for the activation capacity of anti-neutrophil cytoplasmic antibodies in Wegener's granulomatosis[47]. Collectively, these data suggest that the FcγRIIa polymorphism constitutes a risk factor that has pathophysiological importance for IC disorders.

Recent work demonstrating the inability of anti-platelet antibodies to induce thrombocytopenia in FcR γ-chain-deficient mice has solidified and extended the role of FcγRs in type II hypersensitivity disorders (AIHA and ITP)[16]. Corticosteroids, often the first line of treatment for ITP, have suppressive effects on FcγR functions[9], impeding the destruction of antibody-coated platelets by FcγR$^+$ cells of the mononuclear phagocyte system. Other treatments for ITP include intravenous immunoglobulin (IVIg) and anti-Rhesus factor antibody (WinRho). One proposed mechanism of action for IVIg and WinRho suggests that their binding to FcγRs on mononuclear phagocytes leads to inhibition of the Fc-mediated destruction of antibody-coated platelets[48,49]. Decreased FcR function in monocytes derived from IVIg-treated patients, and successful treatment of ITP by infusion of the Fc portion of IgG, support the idea that FcγR blockade is a relevant mechanism of action[50]. A role for FcγRs in AIHA is further supported by prolonged IC clearance in mice treated with an anti-murine FcγRII/III mAb (2.4G2)[16], and delayed clearance of antibody-opsonized erythrocytes in chimpanzees infused with an anti-FcγRIII mAb (3G8)[51]. Furthermore, an ITP patient treated with mAb 3G8 showed a dramatic, albeit transient, rise

in platelet count[53]. Treatment of an IVIg-refractory ITP patient with an anti-FcγRI mAb (197), which triggers downmodulation of FcγRI, showed significant clinical improvement[54]. Although the platelet count remained stable during the five-day mAb treatment, the patient showed a marked rise in platelets in response to subsequent IVIg treatments. A humanized anti-FcγRI mAb (H22)[35] can efficiently downmodulate FcγRI on monocytes and macrophages, resulting in inhibition of phagocytosis and ADCC of antibody-coated cells (P.K. Wallace, unpublished). Clinical trials of this reagent for evaluation of *in vivo* efficacy in ITP and AIHA patients are expected to commence soon.

Concluding remarks

FcγRs are clinically relevant trigger molecules on both myeloid and lymphoid effector cells, and their activation and deactivation can be exploited to combat various diseases. Recently, the signal transduction pathways of FcγRs have been partially delineated and FcγR-specific mAbs and BSMs are being tested in preclinical and clinical studies with encouraging results. Novel techniques to affect directly the intracellular signaling cascade of FcγRs, and multispecific molecules that can simultaneously activate or deactivate several classes of FcγRs, may offer additional therapeutic options.

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References

1 van de Winkel, J.G.J. and Capel, P.J.A. (eds) (1996) *Human IgG Fc Receptors*, R.G. Landes Company
2 Ravetch, J.V. and Kinet, J-P. (1991) *Annu. Rev. Immunol.* 9, 457–492
3 Masuda, M. and Roos, D. (1993) *J. Immunol.* 151, 7188–7195
4 Van den Herik-Oudijk, I.E., Ter Bekke, M.W.H., Tempelman, M.J., Capel, P.J.A. and van de Winkel, J.G.J. (1995) *Blood* 86, 3302–3307
5 Amigorena, S., Bonnerot, C., Drake, J.R. *et al.* (1992) *Science* 256, 1808–1812
6 Parren, P.W.H.I., Warmerdam, P.A., Boeije, L.C. *et al.* (1992) *J. Clin. Invest.* 90, 1537–1546
7 Ory, P.A., Goldstein, I.M., Kwoh, E.E., Clarkson, S.B. and Goldstein, I.M. (1989) *J. Clin. Invest.* 84, 1688–1691
8 de Haas, M., Koene, H.R., Kleijer, M. *et al.* (1996) *J. Immunol.* 156, 2948–2955
9 Salmon, J.E., Millard, S.S., Brogle, N.L. *et al.* (1995) *J. Clin. Invest.* 95, 2877–2885
10 Jones, S.L. and Brown, E.J. (1996) in *Human IgG Fc Receptors* (van de Winkel, J.G.J. and Capel, P.J.A., eds), pp. 149–163, R.G. Landes Company
11 Takai, T., Ono, M., Hikida, M., Ohmori, H. and Ravetch, J.V. (1996) *Nature* 379, 346–349
12 Wallace, P.K., Valone, F.H. and Fanger, M.W. (1995) in *Bispecific Antibodies* (Fanger, M.W., ed.), R.G. Landes Company
13 Liu, C., Goldstein, J., Graziano, R.F. *et al.* (1996) *J. Clin. Invest.* 98, 2001–2007
14 Heijnen, I.A.F.M., van Vugt, M.J., Fanger, N.A. *et al.* (1996) *J. Clin. Invest.* 97, 331–338
15 Sylvestre, D.L. and Ravetch, J.V. (1994) *Science* 265, 1095–1098
16 Clines, R. and Ravetch, J.V. (1995) *Immunity* 3, 21–26
17 Hazenbos, W.L.W., Gessner, J.E., Hofhuis, F.M.A. *et al.* (1996) *Immunity* 5, 181–186
18 Dyer, M.J.S., Hale, G., Hayhoe, F.G.J. and Waldmann, H. (1989) *Blood* 73, 1431–1439
19 Maloney, D.G., Liles, T.M., Czerwinski, D.K. *et al.* (1994) *Blood* 84, 2457–2466
20 Repp, R., Valerius, T., Sendler, A. *et al.* (1991) *Blood* 78, 885–889
21 Berends, D., van der Kwast, T.H., de Both, N.J. and Mulder, P.G. (1989) *Cancer Immunol. Immunother.* 28, 123–130
22 Adams, D.O., Hall, T., Steplewski, Z. and Koprowski, H. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 3506–3510
23 Takahashi, H., Nakada, T., Nakaki, M. and Wands, J.R. (1995) *Gastroenterology* 108, 172–182
24 Dealkens, E.Y., Badger, C.C., Ledbetter, J.A. and Bernstein, I.D. (1985) *J. Immunol.* 135, 2183–2189
25 Riethmueller, G., Schneider-Gadicke, E., Schlimok, G. *et al.* (1994) *Lancet* 343, 1177–1183
26 Baselga, J., Tripathy, D., Mendelsohn, J. *et al.* (1996) *J. Clin. Oncol.* 14, 737–744
27 Elsasser, D., Valerius, T., Repp, R. *et al.* (1996) *Blood* 87, 3803–3812
28 Weiner, L.M., Holmes, M., Adams, G.P., LaCreta, F., Watts, P. and Garcia de Palazzo, I. (1993) *Cancer Res.* 53, 94–100
29 Hombach, A., Jung, W., Pohl, C. *et al.* (1993) *Int. J. Cancer* 55, 830–836
30 Valone, F.H., Kaufman, P.A., Guyre, P.M. *et al.* (1995) *J. Clin. Oncol.* 13, 2281–2292
31 Repp, R., Valerius, T., Wieland, G. *et al.* (1995) *J. Hematother.* 4, 415–421
32 Ball, E.D., Guyre, P.M., Mills, L., Fisher, J., Dinces, N.B. and Fanger, M.W. (1992) *J. Hematother.* 1, 85–94
33 Weiner, L.M., Clark, J.I., Davey, M. *et al.* (1995) *Cancer Res.* 55, 4586–4592
34 Tyler, D.S., Nastala, C.L., Stanley, S.D. *et al.* (1989) *J. Immunol.* 142, 1177–1182
35 Sanders, L.A.M., Feldman, R.G., Voorhorst-Ogink, M.M. *et al.* (1995) *Infect. Immun.* 63, 73–81
36 Salmon, J.E., Edberg, J.C., Brogle, N.L. *et al.* (1992) *J. Clin. Invest.* 89, 1276–1281
37 Erbe, D.V., Pfefferkorn, E.R. and Fanger, M.W. (1991) *J. Immunol.* 146, 3145–3151
38 Mady, B.J., Kurane, I., Erbe, D.V. *et al.* (1991) *J. Immunol.* 147, 3139–3144
39 Connor, R.I., Dinces, N., Howell, A.L., Romet-Lemonne, J.L., Pasquali, J.L. and Fanger, M.W. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 9593–9597
40 Frank, M.M., Hamburger, M.I., Lawley, T.J., Kimberly, R.P. and Plotz, P.H. (1979) *New Engl. J. Med.* 300, 518–523
41 Salmon, J.E., Kimberly, R.P., Gibofsky, A. and Fotino, M. (1984) *J. Immunol.* 133, 2525–2531
42 Boros, P., Odin, J.A., Muryoi, T., Masur, S.K., Bona, C. and Unkeless, J.C. (1991) *J. Exp. Med.* 173, 1473–1482

43 Bona, P., Muryoi, T., Spiera, H., Bona, C. and Unkeless, J.C. (1993) *J. Immunol.* 150, 2018–2024
44 Inzinc, P.L., Powell, M.S., McKenzie, I.F.C. and Hogarth, P.M. (1993) *J. Exp. Med.* 178, 1617–1628
45 Duits, A.J., Bootsma, H., Derksen, R.H.W.M. *et al.* (1995) *Arthritis Rheum.* 38, 1832–1836
46 Salmon, J.E., Millard, S., Schachter, L.A. *et al.* (1996) *J. Clin. Invest.* 97, 1348–1354
47 Porges, A.J., Redecha, P.B., Kimberly, W.T., Csernok, E., Gross, W.L. and Kimberly, R.P. (1994) *J. Immunol.* 153, 1271–1280
48 Imbach, P., Barandun, S., d'Apuzzo, V. *et al.* (1981) *Lancet* i, 1228–1231
49 Bussel, J.B., Kimberly, R.P., Inman, R.D. *et al.* (1991) *Blood* 77, 1884–1893
50 Debre, M., Bonnet, M-C., Fridman, W-H. *et al.* (1993) *Lancet* 342, 945–949
51 Kurlander, R.J., Ellison, D.M., Hall, J. *et al.* (1984) *J. Immunol.* 133, 855–862
52 Clarkson, S.B., Kimberly, R.P., Valinsky, J.E. *et al.* (1986) *J. Exp. Med.* 164, 474–489
53 Clarkson, S.B., Bussel, J.B., Kimberly, R.P. *et al.* (1986) *New Engl. J. Med.* 314, 1236–1239
54 Ericson, S.G., Coleman, K.D., Wardwell, K. *et al.* (1996) *Br. J. Haematol.* 92, 718–724
55 Cazzimo, R.P., Tempest, P.R., White, R. *et al.* (1995) *J. Immunol.* 155, 4996–5002
56 Cenac, P.C., Imric, J.G., Scott, A.M. *et al.* (1994) *Blood* 83, 1760–1768
57 Goldstein, J., Cruziano, R.F., Sunderpandiyan, K., Somasundaram, C. and Dao, Y.M. *J. Immunol.* (in press)

Immunoisolation: at a turning point

Robert P. Lanza and William L. Chick

The principle of immunoisolation is to separate transplanted cells from the hostile immunological environment of the host by a selectively permeable membrane. Low-molecular-weight substances such as nutrients, electrolytes, oxygen and biotherapeutic agents are exchanged across the membrane, while immunocytes, antibodies and other transplant-rejection effector mechanisms are excluded. Here, Robert Lanza and William Chick review these systems.

Immunoisolation is the technological key to enabling cell and tissue transplants to resist immune attack, and raises the possibility of xenotransplantation without the need for immunosuppressive drug therapy. In the USA alone, 400 billion dollars are spent each year caring for patients who suffer tissue loss or dysfunction and for whom human tissue transplant material is in short supply. It is clear that the pressure to transplant animal tissues into humans will grow and inversely. Although a deeper understanding of the immune system and the immune rejection process may one day lead to the development of therapies that overcome the vigorous humoral and cellular immune responses associated with the transplantation of xenogeneic tissues, many of these solutions remain years or decades in the future. Immunoisolation offers a practical means for solving this problem in a reasonable time-frame and may prove the only realistic way to establish prolonged survival of cell xenografts. Cells and tissues offer an advantage in this respect: they can be maintained and manipulated more easily than whole organ grafts, resulting in a more effective therapy.

Immunoisolation has broad application to treating major diseases such as diabetes, as well as a wide range of other disorders. These applications predict the use of a variety of cells, such as hepatocytes for the treatment of liver failure, chromaffin cells for chronic pain, cells that produce clotting factors for hemophilia, and nerve growth factors for neurodegenerative disorders such as Parkinson's and Alzheimer's disease. Moreover, by using recombinant DNA and cell-engineering technologies, it should also prove possible to treat patients suffering from other disorders, including immunodeficiencies and cancer. To date, however, most of the research in the area of immunoisolation has been carried out with pancreatic islets. In patients with insulin-dependent type I diabetes mellitus (IDDM), there is a marked decrease in the number of β cells in the pancreas. There is hope that the transplantation of islets will not only eliminate the need for daily insulin injections, but will also prove effective in preventing or retarding the development of complications associated with the disease. Unfortunately, studies indicate that transplanted islets are exquisitely sensitive both to conventional rejection and to damage by autoimmune activity specifically directed against the β cells. Local cytokine release is toxic to islet cells and inhibits normal insulin secretion by any cells that are not lethally affected.

The currently limited supply of human pancreases, and the fact that multiple glands may be required to isolate sufficient numbers of islets to treat a single patient, indicate that techniques must be further developed and refined for xenografting of isolated islets from animal sources to diabetic patients. Such techniques must overcome

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Cross-linking of monocyte plasma membrane Fcα, Fcγ or mannose receptors induces TNF production

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SUMMARY

We have studied and compared the effects of IgA and IgG immune complexes and concanavalin A (Con A) on human monocyte tumour necrosis factor (TNF) production. The presence of IgA-containing immune complexes in monocyte monolayers resulted in a dose-dependent increase of TNF production. Similar results were obtained with IgG-containing immune complexes and Con A. The presence of monomeric IgA or IgG did not increase TNF secretion. Both IgA and IgG immune complexes also increased monocyte interleukin-1β (IL-1β) production. Galactose inhibited the effect of IgA but not IgG immune complexes, while mannose inhibited the effect of Con A. Prednisolone abrogated TNF production, while indomethacin enhanced TNF production in all instances where cross-linking of plasma membrane receptors was achieved. These results indicate that activation of Fcα receptors (FcαR), FcγR or mannose receptors of the human monocyte plasma membrane by cross-linking results in increased TNF and IL-1β secretion. These findings may be of particular relevance in the pathogenesis of IgA immune complex-mediated disease.

INTRODUCTION

Immune complex (IC) deposition in target organs is implicated in the pathogenesis of tissue injury in a wide variety of autoimmune diseases, chronic arthritis and glomerulonephritis.[1,2] Part of the inflammatory response is attributed to binding of these complexes to the Fc receptors of local tissue macrophages. The stimulated phagocytes secrete a variety of inflammatory products including prostaglandins, leukotrienes, procoagulant factors, cytokines, neutral and lysosomal enzymes.[3-7] In these diseases, particularly in chronic glomerulonephritis, increased numbers of monocytes have been demonstrated in the tissue lesion as part of the cellular infiltrate.[8-10]

There is increasing recognition that immune complexes of IgA isotype play a pathogenic role in glomerulonephritis, such as IgA nephropathy and Henoch Schonlein purpura.[11] In fact, the former is now recognized as the commonest form of chronic glomerulonephritis.[12] In addition, IgA immune complexes have also been implicated in chronic rheumatoid disease, particularly of the juvenile type.[13] The presence of Fcα receptors (FcαR) on human peripheral blood monocyte plasma membrane has been demonstrated by E-IgA rosette formation, indirect immunofluorescence and competitive inhibition of binding of radiolabelled ligand.[14-17] Recently, a 60,000 MW FcαR, distinct from the three FcγR which bind both monomeric and polymeric forms of IgA1 and IgA2, has been defined[18,19] and a cDNA clone encoding for this receptor has been characterized.[20] Evidence for FcαR on human polymorphonuclear leucocytes, rat peritoneal macrophages and human breast milk macrophages has also been presented.[21-23]

The cytokine tumour necrosis factor-α (TNF-α) is primarily a product of mononuclear phagocytes that originally was shown to have cytotoxic properties against tumour cells.[24] However, this monokine has many systemic effects including mediation of the septic shock syndrome, induction of cachexia, fever, the acute phase response and inhibition of lipoprotein lipase.[25,26] In addition, this cytokine induces complement gene expression and increased biosynthesis of several complement proteins by hepatocyte, macrophage and fibroblast cultures.[26,27] TNF also promotes macrophage oxidative burst and increases the killing of intracellular organisms by induction of non-oxidative mechanisms.[28-30] Apparent opposing effects of TNF have been shown in *in vivo* models of disease in which TNF inhibits insulitis and autoimmune diabetes mellitus and lupus glomerulonephritis in experimental animals.[30-32] In animals it enhances host resistance *in vivo* to parasites and intracellular infections; however, it is central in the inflammatory response in bacterial meningitis.[33-35] We have studied the effects of immune complexes of the IgA isotype on monocyte production of TNF. These results have been compared to the effects of binding of concanavalin A (Con

Abbreviations: Con A, concanavalin A; FcαR, Fcα receptor; FcγR, Fcγ receptor; IC, immune complexes; IL-1β, interleukin-1β; sIgA, secretory IgA; TNF, tumour necrosis factor.

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A) to its known mannose receptor on the monocyte plasma membrane and also cross-linking of FcαR and FcγR.

MATERIALS AND METHODS

Monocyte cultures

Monocyte monolayers were prepared as described elsewhere.[36] In brief, human donor heparinized blood was centrifuged at 400 *g* for 10 min and the plasma and the buffy coat layer were removed. The white cell suspension was layered on Ficoll–Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) centrifuged at 400 *g* for 20 min at room temperature, and the mononuclear cell layer was removed from the interface. These cells were washed three times in Hanks' balanced salt solution, and were resuspended in RPMI-1640 medium (Microbiological Associates, Betheseda, MD) supplemented with 10% heat-inactivated Millipore-filtered fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco, Grand Island, NY). The number of monocytes and lymphocytes in each preparation was determined by morphology of Giemsa-stained cytocentrifuge preparations and staining for non-specific esterase. The cell concentration was adjusted to 1×10^6 monocytes/ml, and aliquots of 0·2 ml (2×10^5 monocytes) were pipetted into 96-well flat-bottomed tissue culture trays, 8-mm diameter, or 0·5 ml (5×10^5 monocytes) into 24-well flat-bottomed tissue culture trays (Falcon, Oxnard, CA).

Adherence of monocytes was facilitated by gentle rocking at 37° for 45 min, after which time the non-adherent cells were removed by washing vigorously three times. Giemsa staining of the adherent cell population identified them as 90–95% monocytes. These cell cultures were maintained in complete medium at 37° in a humidified atmosphere of 5% CO_2.

Assays for cytokines

TNF activity was determined by lysis of the mouse L-929 cell line.[31] The cell cultures were plated in 96-well tissue culture trays (4×10^4 cells/well), treated with actinomycin-D 2 μg/ml for 1 hr and viability was determined by uptake of crystal violet after incubation in the presence of the monocyte extracellular medium for 18 hr. The effect of recombinant human TNF-α, ranging from 5 to 10,000 units/ml, was included in each assay as a standard curve. Rabbit anti-TNF-α antibody added to extracellular samples completely inhibited the effect of lysis of this cell line. In addition, in selected experiments the TNF concentrations determined by this biological assay were compared to those of a commercial ELISA assay and were strongly correlated (results not shown).

The interleukin-1β (IL-1β) concentration was determined in the extracellular medium by radioimmunoassay (Advanced Magnetics Incorporated, Cambridge, MA). Aliquots of 100 μl of the samples were tested and the assay was performed according to the instructions of the commercial source. An IL-1β standard was included in each assay and results were calculated from the standard curve which ranged from 0 to 500 units/ml.

AB positive serum from a single donor was used in all these experiments. Concentrations of IgA and IgG in this serum were 100 mg/dl and 800 mg/dl, respectively. Human IgG, secretory IgA (sIgA) and respective goat anti-human immunoglobulin antibodies were obtained from Bio Yeda, Rehovat, Israel. $F(ab')_2$ fragments of these antibodies were prepared by pepsin digest. Concanavalin A (Con A), succinyl Con A, polymyxin B, mannose, galactose and lipopolysaccharide (LPS) from *Escherichia coli* 026;B6 were purchased from Sigma Chemical Co. (St Louis, MO) and made up at the designated concentrations for use.

Immune complexes

Immune complexes containing human immunoglobulin of either the IgG, IgM or IgA isotype were prepared *in situ* by adding the respective goat anti-human $F(ab')_2$ antibody to confluent monocyte monolayers cultured in RPMI-1640 with 15% human AB serum in the presence of polymyxin B sulphate (5 μg/ml). In order to exclude the possible effects of activated complement components, AB serum was heat inactivated at 56° for 30 min. The binding of IgA and IgG immune complexes to the monocyte membrane was confirmed by indirect immunofluorescence using a rabbit anti-goat IgG fluorescein-labelled second antibody. Immunohistology showed that a rim of fluorescence was present when incubations were carried out at 0°, while incubation at 37° showed patching on the membrane after 30 min and the cells had internalized most of the labelled antibody after 2 hr (results not shown).

In addition, preformed immune complexes were prepared at 4° by incubating purified sIgA preparations with antibody at varying concentrations and allowing precipation to occur. These preformed complexes were then added at varying concentrations to the monocyte monolayer. Immune complexes were also formed *in situ* by adding increasing concentrations of sIgA to monocyte monolayers in fetal calf serum and 30 min thereafter the $F(ab')_2$ antibody was added.

Preliminary experiments showed that addition of endotoxin to human monocyte monolayers resulted in a dose-dependant increase of TNF in the extracellular medium. The effect of endotoxin was completely inhibited by the presence of polymyxin B (5 μg/ml) in the medium (results not shown). Therefore, in order to exclude the effect of contaminating endotoxin, all subsequent experiments were carried out with polymyxin B in the culture medium.

Statistical analysis

The results are expressed in the figures and tables as mean ± SD. Significant effect of addition of immune complexes by comparison to control cultures was tested by paired Student's *t*-test.

RESULTS

Effect of Con A on TNF production by human monocytes in culture

Con A resulted in a dose-dependent increase of TNF production (Table 1) which was not altered by polymyxin B. The presence of a Con A stimulus for 2 hr was sufficient to result in a significant increase of TNF production compared to untreated cultures (results not shown). Prior incubation of the monocyte monolayer with mannose inhibited Con A-induced TNF secretion, while succinyl Con A resulted in only a slight increase in TNF production compared to Con A (Table 1).

Effect of IgA and IgG immune complexes on human monocyte TNF production

Monocytes from different human donors varied considerably in the amount of basal TNF production and in response to the

Table 1. Effect of mannose on Con A-induced monocyte TNF production

Experimental condition		TNF (ng/ml/10^5 cells)
Control	—	0·4±0·1
Mannose (1×10^{-5} M)	—	0·4±0·1
Con A	0·1 (μg/ml)	0·4±0·1
Con A	1·0 (μg/ml)	0·6±0·1
Con A	2·0 (μg/ml)	11·5±3·2
Con A	5·0 (μg/ml)	77·6±4·8
Con A	10·0 (μg/ml)	166·7±9·3
Con A (10 μg/ml)+mannose	(1×10^{-5} M)	1·7±0·1
Succinyl Con A	10·0 (μg/ml)	14·3±1·0

Results are the mean ±SD of two different experiments done in triplicate. The mannose (10^{-5} M) was added 30 min prior to Con A (10 μg/ml) and the monolayers were then washed twice after 1 hr. The cells were maintained in culture thereafter for 24 hr and the extracellular material was harvested for determination of TNF concentrations. Experiments were done in the presence of polymixen B (5 μg/ml) in the culture medium.

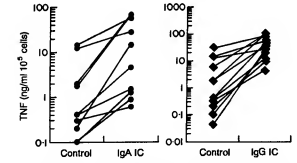

Figure 1. Comparison of monocyte TNF production following cross-linking of FcγR or FcαR. Activation of the respective receptors was effected by cross-linking of the ligand in the AB serum. Extracellular medium was harvested 24 hr after addition of the stimulus for determination of TNF concentration. The varied response of different donors is illustrated. Each point is the average of triplicate cultures and represents experiments performed on different days.

various stimuli. However, cross-linking of either the FcαR or the FcγR, which was achieved by addition of goat $F(ab')_2$ antibody to the respective immunoglobulin, resulted in a consistent increase in TNF production (Fig. 1). Decreasing the amount of antibody added to the extracellular medium resulted in a dose-dependent decrease in TNF production (results not shown). Similar formation of IgM complexes had no effect. Immune complexes of the IgG isotype resulted in an approximately five-fold greater amount of TNF secretion compared to the effect of IgA immune complexes. Cumulative data of 9–15 experiments showed that IgG immune complexes resulted in a 101·0+56·3 SEM-fold increase; while IgA immune complexes resulted in a 19·6+7·3 SEM-fold increase. Both instances were significantly increased ($P<0.001$) by comparison to control cultures, as calculated by Student's paired *t*-test.

Incubation of the monocyte monolayers in the presence of immune complexes of both IgA and IgG isotype resulted in a

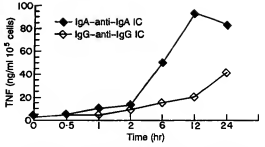

Figure 2. Progressive increase in monocyte TNF production following cross-linking of either FcγR or FcαR. Results are from a representative experiment, where each time-point was done in triplicate.

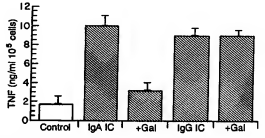

Figure 3. Addition of galactose (Gal) (1×10^{-4} M) prior to cross-linking of FcγR and FcαR. Galactose was added 1 hr prior to the respective goat $F(ab')_2$ antibody. One hour later the monolayers were washed twice. The extracellular medium was harvested 24 hr later and assayed for TNF biological activity. All results are the mean ± SD of two experiments, each done in triplicate.

progressive increase in TNF production (Fig. 2). Exposure of the monocyte monolayer to either IgA or IgG complexes for 30 min and then subsequent washing was sufficient to induce an increase in monocyte TNF production (results not shown). The second antibody effecting the cross-linking was not responsible for the increased monocyte TNF production; as addition of these goat antibodies to monocyte monolayers in the presence of fetal calf serum rather than AB serum did not induce TNF production.

In order to test specificity of activitation via FcαR, we examined the effect of prior addition of galactose to the monocyte monolayers. We used concentrations of galactose which we previously had shown to inhibit binding of sheep red blood cells coated with IgA to human monocytes.[23] IgA immune complex-induced TNF production was inhibited by the presence of galactose (1×10^{-4} M); however, no effect of galactose on the IgG immune complex-induced increase in monocyte TNF production was observed (Fig. 3). Similarly, prior addition of mannose to monocyte monolayers inhibited Con A-induced monocyte TNF production (Table 1).

Effect of preformed IgA complexes on monocyte TNF production

These experiments were performed in the presence of fetal calf serum. A dose-dependent effect following addition of preformed IgA immune complexes on monocyte TNF secretion could be demonstrated (Fig. 4a). In addition, soluble immune complexes,

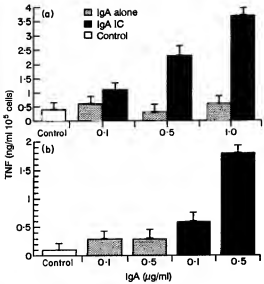

Figure 4. Dose-dependent increase in TNF production following addition of IgA anti-IgA immune complexes. (a) Preformed complexes were formed following precipation of sIgA and anti-IgA antibody. These complexes were then added at varying concentrations to the monocyte monolayer. Extracellular medium was harvested following 24 hr incubation period and used for TNF determinations. (b) Immune complexes were formed *in situ* by incubating the monocyte monolayers in fetal calf serum. Increasing concentrations of sIgA was added and 30 min thereafter the $F(ab')_2$ antibody was added. Extracellular medium was harvested following a 24 hr incubation period and used for TNF determination.

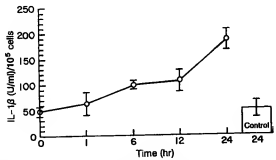

Figure 5. Kinetics of monocyte IL-1β production following cross-linking of FcγR. Results are of a representative experiment performed in duplicate cultures. Extracellular medium from each well was assayed in duplicate for IL-1β concentration by radioimmunoassay. IgA complexes were formed by addition of the $F(ab')_2$ goat antibody.

which were formed by the addition of various concentrations of human sIgA and 30 min thereafter goat $F(ab')_2$ anti-IgA added to the monolayer culture, also resulted in a dose-dependent increase of monocyte TNF production (Fig. 4b).

Effect of IgA immune complexes on human monocyte IL-1β production

IgA immune complexes formed by the addition of goat $F(ab')_2$ anti-human IgA antibody in the presence of AB serum resulted

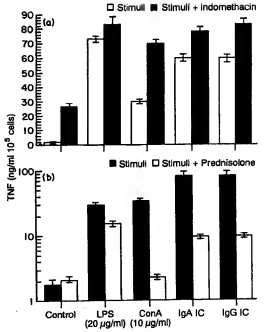

Figure 6. (a) Increase in TNF production by human monocyte monolayers following co-incubation of various stimuli with indomethacin (5 μg/ml). The extracellular medium was harvested after 24 hr incubation and used for TNF determinations. (b) Inhibition of monocyte monolayer TNF production by corticosteroids with various stimuli. Prednisolone (2×10^{-5} M) was added 4 hr prior to addition of the stimuli. The extracellular medium was harvested after 24 hr incubation and used for TNF determinations. Each of the above figures represents two different experiments; where each of the conditions was performed in triplicate.

in a dose-dependent increase in monocyte IL-1β production (Fig. 5). Similar results were obtained with IgG immune complexes (results not shown).

Effect of indomethacin or prednisolone on TNF production

Stimulation of TNF production by addition of either Con A, IgA or IgG complexes or endotoxin was increased in each instance when indomethacin (5 μg/ml) was added to the cell culture (Fig. 6a). Conversely, incubation of the cell cultures with the various stimuli in the presence of prednisolone (2×10^{-5} M) inhibited TNF production (Fig. 6b).

DISCUSSION

In these experiments we have shown that binding of IgA immune complexes to monocyte monolayers results in increased monocyte TNF production. Immune complexes were formed either by addition of goat $F(ab')_2$ anti-human IgA antibodies in the presence of AB serum or by adding the goat $F(ab')_2$ anti-human IgA to varying concentrations of sIgA in fetal calf serum. Neither the presence of sIgA nor antibody alone had any effect. Addition of preformed IgA immune complexes to monocyte monolayers also achieved similar responses. Formation of IgG complexes in similar fashion also resulted in increased monocyte TNF production. These latter findings of cross-linking of FcγR confirm results reported by Debets *et al.*[36]

In addition, both IgA and IgG immune complex formation resulted in increased monocyte IL-1β production. The latter finding has also recently been reported by Chantry *et al.*[37] The specificity of activation via FcαR was confirmed as abrogation of IgA complex-induced TNF production could be effected by prior incubation of the monocyte monolayers with galactose, while this was not apparent with IgG complexes. These findings are probably due to the particularly rich galactose residues in the Fc portion of the IgA molecule, thus resulting in inhibition of binding to the receptor by the increased concentration of galactose in the medium.[38] Increased monocyte TNF production was also induced by addition of Concanavalin A, which binds to the macrophage mannose receptor.[39] Succinyl Con A which does not result in cross-linking of the receptor only had a minimal effect and inhibition of Con A-induced effects was achieved with mannose.

Monocyte/macrophage FcγR mediate a large spectrum of functions including phagocytosis and endocytosis of IgG-coated particles, antigen presentation and antibody cytotoxicity. Previous studies have shown that activation of monocytes via their FcγR by immune complexes or Fc fragments has resulted in increased prostaglandin E2, leukotriene and collagenase and recently IL-1β secretion has also been demonstrated.[3-5,37,40] Similarly, binding and phagocytosis of particles via FcαR has been demonstrated and activation of the FcαR increases monocyte prostaglandin E_2 (PGE_2) production and the oxidative burst.[17,21] We have also shown, using similar experimental conditions, that IgA complexes result in increased macrophage biosynthesis of C3 (J. Laufer *et al.*, manuscript submitted for publication). The demonstration of increased TNF and IL-1β production adds to the array of inflammatory products produced consequent on activation of the monocyte FcαR.

The infiltrating macrophages during the course of glomerulonephritis may not only be directly responsible for secretion of their inflammatory products, but they also express class II antigen epitopes and are capable of eliciting an MHC-restricted cellular immune response.[41] The possibility that activation of IgA receptors of the mononuclear phagocytes occurs in Berger's disease is suggested by the recently recognized clinicopathological association of the number of monocyte/macrophages per glomerulus and the number of glomerular crescents and the degree of proteinuria.[42]

TNF may exert its inflammatory potential locally on other cell types, for example fibroblasts to increase their inflammatory response (complement, PGE_2) or induce HLA class I and II antigens and leucocyte adhesion molecules, and thus may be involved in enhancing the local cellular response, or it may act in an autocrine fashion on the macrophage.[30]

The demonstration of increased TNF production in all experimental conditions in the presence of indomethacin, confirms that PGE_2 that is secreted concomitantly using these experimental conditions, down-regulates TNF production.[43] Nevertheless, there is increasing evidence demonstrating increased TNF gene expression in the target organs of these immune complex-mediated diseases.[44] Corticosteroids, such as prednisolone, had an inhibitory effect on the immune complex-induced TNF secretion, confirming that this mechanism of action may be of therapeutic benefit.[45]

There is now considerable evidence that immune complexes are formed *in situ* in chronic immune complex disease rather than by simple deposition of circulating immune complexes.[1] We have also shown that in murine models of autoimmune lupus nephritis, complement mRNA of C3 increases with progression of disease.[46,47] The present studies indicate that immune complexes, particularly of IgA isotype may exert their inflammatory effect via local cytokine production. Confirmation of this occurrence, at least in human IgA-mediated disease, and demonstration of the predominant cell responsible for TNF-γ or IL-1β production, will be possible with *in situ* hybridization techniques of affected tissue. The net effect of the cytokine TNF production, that is whether it enhances or abrogates the inflammatory response in these lesions, has not been elucidated.

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REFERENCES

1. Couser W.G. (1985) Mechanisms of glomerular injury in immune complex disease. *Kidney Int.* **28**, 569.
2. Krane S.M. & Simon L.S. (1986) Rheumatoid arthritis: clinical features and pathogenic mechanisms. *Med. clin. North. Am.* **70**, 263.
3. Passwell J.H., Dayer J.M. & Merler E. (1979) Increased prostaglandin production by human monocytes following membrane receptor activation. *J. Immunol.* **123**, 115.
4. Passwell J.H., Dayer J.M., Gass R. & Edelson P.J. (1980) Regulation of the secretion of collaganese, PGE_2 and lysozyme by mouse peritoneal macrophages. *J. Immunol.* **125**, 910.
5. Rouzer C.A.M., Scott W.A., Hamill A.L. & Cohn Z.A. (1980) Dynamics of leukotriene C production by macrophages. *J. exp. Med.* **152**, 1594.
6. Wiggins R.C., Glatfelter A. & Brukman J. (1985) Procoagulant activity in glomeruli and urine of rabbits with nephrotoxic nephritis. *Lab. Invest.* **53**, 156.
7. Ragsdale C.G. & Arend W.P. (1979) Neutral proteinase secretion by human monocytes. *J. exp. Med.* **149**, 954.
8. Holdsworth S.R., Neale T.J. & Wilson C.B. (1981) Abrogation of macrophage-dependent injury in experimental glomerulonephritis in the rabbit. *J. clin. Invest.* **68**, 686.
9. Kimura M., Nagase M., Hishida A. & Honda N. (1987) Intramesangial passage of mononuclear phagocytes in murine lupus glomerulonephritis. *Am. J. Path.* **127**, 161.
10. Boyce N.W. & Holdsworth S.R. (1986) Quantitation of the intrarenal uptake of immunoglobulin aggregates by macrophages in diffuse proliferative glomerulonephritis. *Clin. exp. Immunol.* **64**, 638.
11. Emancipator S.N., Gallo G.R. & Lamm M.E. (1985) IgA nephropathy: perspective on pathogenesis and classification. *Clin. Nephrol.* **24**, 161.
12. Emancipator S.N. & Lamm M.E. (1989) IgA nephropathy: pathogenesis of the most common form of nephritis. *Lab. Invest.* **60**, 168.
13. Czerkinsky C., Koopman W.J. & Jackson S. (1986) Circulating immune complexes and immunoglobulin A rheumatoid factor in patients with mesangial immunoglobulin A nephropathies. *J. clin. Invest.* **77**, 1931.
14. Fanger M.W., Pugh J. & Bernier G.M. (1981) The specificity of receptors for IgA on human peripheral polymorphonuclear cells and monocytes. *Cell. Immunol.* **60**, 324.
15. Fanger M.W., Shen L., Pugh J. & Bernier G.M. (1980) Subpopulations of human peripheral granulocytes and monocytes express receptors for IgA. *Proc. natl. Acad. Sci. U.S.A.* **77**, 3640.
16. Chevailler A., Monteiro R.C., Kubagawa H. & Cooper M.D. (1989) Immunofluorescence analysis of IgA binding by human

mononuclear cells in blood and lymphoid tissue. *J. Immunol.* **142,** 2244.
17. Paden S., Jaffe C.L. & Passwell J.H. (1991) Activation of human monocytes via their sIgA receptors. *Immunology*, **72,** 188.
18. Monteiro R.C., Kubagawa H. & Cooper M.D. (1990) Cellular distribution, regulation and biochemical nature of an IgA Fc receptor in humans. *J. exp. Med.* **1171,** 597.
19. Monteiro R.C., Cooper M.D. & Kubagawa H. (1992) Molecular heterogenicity of Fcα receptors detected by receptor-specific monoclonal antibodies. *J. Immunol.* **1048,** 1764.
20. Maliszewski C.R., March C.J., Shoenborn M.A., Gimpel S. & Shen L. (1991) Expression cloning of a human Fc receptor for IgA. *J. exp. Med.* **172,** 1665.
21. Gorter A., Hiemstra P.S. & Van der Voort E.A.M. (1987) IgA and secretory IgA opsonized *S. aureus* induce a respiratory burst and phagocytosis by polymorphonuclear leukocytes. *Immunology*, **61,** 303.
22. Gorter A., Hiemstra P.S., Van der Voort E.A.M., Van Es, & Daha M.R. (1988) Binding of human IgA to rat peritoneal macrophages. *Immunology*, **64,** 207.
23. Robinson G., Volovitz B. & Passwell J.H. (1991) sIgA receptors on human breast milk macrophages. *Pediat. Res.* **29,** 429.
24. Beutler B., Cerami A. & Cachectin. (1987) More than a tumor necrosis factor. *N. Engl. J. Med.* **316,** 379.
25. Beutler B.A., Mahoney J., Le Trang N., Pekala P. & Cerami A. (1988) Purification of cachectin, a lipoprotein lipase-suppressing hormone secreted by endotoxin-induced RAW 264:7 cells. *J. exp. Med.* **161,** 984.
26. Perlmutter D.H., Dinarello C.A., Punsal P.I. & Colten H.R. (1986) Cachectin/tumor necrosis factor regulates hepatic acute-phase gene expression. *J. clin. Invest.* **78,** 1349.
27. Perlmutter D.H. & Colten H.C. (1986) Molecular immunobiology of complement biosynthesis: A model of single-cell control of effector-inhibitor balance. *Annu. Rev. Immunol.* **4,** 231.
28. Drapier J.C., Wietzerbin J. & Hibbs J.B. (1988) Interferon-γ and tumour necrosis factor induce the L-arginine dependent cytotoxic effector mechanism in murine macrophages. *Eur. J. Immunol.* **18,** 1587.
29. Liew F.Y., Parkinson C., Millott S., Severn A. & Carrier M. (1990) Tumour necrosis factor (TNF alpha) in leishmaniasis. *Immunology*, 69, 590.
30. Vassalli P. (1992) The pathophysiology of tumour necrosis factors. *Annu. Rev. Immunol.* **10,** 411.
31. Jacob C. & McDevitt H. (1988) Tumour necrosis factor-α in murine autoimmune 'lupus nephritis'. *Nature*, **331,** 356.
32. Jacob C.O., Aiso S., Nichie S.A., McDermott H.O. & Acha Orbea H. (1990) Prevention of diabetes in non obese diabetic mice by tumour necrosis factor (TNF). Similarities between TNF-α and IL-1. *Proc. natl. Acad. Sci. U.S.A.* **87,** 968.
33. Havell E.A. (1989) Evidence that tumour necrosis factor has an important role in antibacterial resistance. *J. Immunol.* **143,** 2894.
34. Clark I.A., Hunt N.H., Butcher G.A. & Cowden W.B. (1989) Inhibition of murine malaria (plasmodium chabaudi) in vivo by recombinant IFN-gamma or tumor necrosis factor and its enhancement by butylated hydroxyanisole. *J. Immunol.* **143,** 127.
35. Leist T.P., Frei K., Kam-Hansen S., Zinkernagel R.M. & Fontana A. (1988) Tumor necrosis factor in cerebrospinal fluid during bacterial, but not viral meningitis. Evaluation in murine model infections and in patients. *J. exp. Med.* **167,** 1743.
36. Debets J.M.H., Van der Linden C.J., Dieteren I.E.M., Leeuwenberg J.F.M. & Buurman W.A. (1988) Fc-receptor cross-linking induces rapid secretion of tumor necrosis factor (Cachectin) by human peripheral blood monocytes. *J. Immunol.* **141,** 1197.
37. Chantry D., Winearls C.G., Maine R.N. & Feldman M. (1989) Mechanism of immune complex-mediated damage: induction of interleukin 1 by immune complexes and synergy with interferon-γ and tumor necrosis factor α. *Eur. J. Immunol.* **19,** 189.
38. Mestecky J. & McGhee J.R. (1987) Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. *Adv. Immunol.* **40,** 153.
39. Ezekowitz R.A.B., Sastry K., Bailly P. & Warner A. (1990) Molecular characterization of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeasts in Cos-1 cells. *J. exp. Med.* **172,** 1785.
40. Fridman W.H., Bonnerot C., Baeron M., Amigorena S., Teiland J.L. & Sauter C. (1992) Structural basis of Fcγ receptor functions. *Immunol. Rev.* **124,** 149.
41. Schreiner G.F., Kiely J.M., Cotran R.S. & Unanue E.R. (1981) Characterization of residual glomerular cells in the rat expressing Ia determinants and manifesting genetically restricted interactions with lymphocytes. *J. clin. Invest* **1981,** 68.
42. Akima S., Nakayama M., Naito M., Sato T. & Tekahashi R. (1991) Significance of mononuclear phagocytes in IgA nephropathy. *Kidney Int.* **39,** 684.
43. Spengler R.N., Spengler M.L., Strieter R.M., Remick D.G., Larrick J.W. & Kunkel S. (1989) Modulation of tumor necrosis factor and gene expression. Desensitization of prostaglandin E2-induced suppression. *J. Immunol.* **142,** 4346.
44. Macnaul K.L., Hutchinson N.L., Parsons J.N., Kahn Bayne E. & Tolli M.J. (1990) Analysis of IL-1 and TNFα gene expression in human rheumatoid synoviocytes and normal monocytes by *in situ* hybridization. *J. Immunol.* **145,** 4145.
45. Szefler S.J., Norton C.E., Ball B., Gross J.M., Aida Y. & Pabst M.J. (1989) IFN-γ and LPS overcome glucocorticoid inhibition of priming for superoxide release in human monocytes: evidence that secretion of IL-1β and tumor necrosis factor is not essential for monocyte priming. *J. Immunol.* **142,** 3985.
46. Passwell J.H., Schreiner G., Nonaka M., Beuscher H.U. & Colten H.R. (1988) Local increase in expression of the complement genes and synthesis of complement proteins in kidneys affected by chronic immune complex disease. *J. clin. Invest.* **82,** 1676.
47. Passwell J.H., Schreiner G., Wetsel R. & Colten H.R. (1990) Expression of the complement genes C2, C3, C4 and factor B in the diseased organs of NZB and NZB × W (F1) inbred mouse strains. *Immunology*, **71,** 290.

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Brief Definitive Report

Reversal of Proinflammatory Responses by Ligating the Macrophage Fcγ Receptor Type I

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Summary

Macrophages can respond to a variety of infectious and/or inflammatory stimuli by secreting an array of proinflammatory cytokines, the overproduction of which can result in shock or even death. In this report, we demonstrate that ligation of macrophage Fcγ receptors (FcγR) can lead to a reversal of macrophage proinflammatory responses by inducing an upregulation of interleukin (IL)-10, with a reciprocal inhibition of IL-12 production. IL-10 upregulation was specific to FcγR ligation, since the ligation of the Mac-1 receptor did not alter IL-10 production. The identification of the specific FcγR subtype responsible for IL-10 upregulation was determined in gene knockout mice. Macrophages from mice lacking the FcR γ chain, which is required for assembly and signaling by FcγRI and FcγRIII, failed to upregulate IL-10 in response to immune complexes. However, mice lacking either the FcγRII or the FcγRIII were fully capable of upregulating IL-10 production, implicating FcγRI in this process. The biological consequences of FcγRI ligation were determined in both in vitro and in vivo models of inflammation and sepsis. In all of the models tested, the ligation of FcγR promoted the production of IL-10 and inhibited the secretion of IL-12. This reciprocal alteration in the pattern of macrophage cytokine production illustrates a potentially important role for FcγR-mediated clearance in suppressing macrophage proinflammatory responses.

Key words: CD64 • macrophage • interleukin 10 • inflammation • Fc receptors

Macrophages are prodigious secretory cells which can produce a number of molecules that can either potentiate or dampen immune responses (1). In response to infectious or inflammatory stimuli, macrophages can produce several proinflammatory molecules, including IL-12, TNF-α, IL-6, and IL-1 (1, 2). These proinflammatory molecules are important for host defense, because experimentally infected animals deficient in these cytokines are more susceptible to acute bacterial infections than are normal animals (3, 4). However, the production of proinflammatory cytokines must be tightly regulated, since their production is also correlated with many of the pathologies associated with acute sepsis or with autoimmune diseases. Macrophages themselves can participate in this regulation by the production of antiinflammatory molecules. The secretion of prostaglandins, TGF-β, and IL-10 by macrophages has been associated with antiinflammatory responses. Thus, the balance between the secretion of pro- and antiinflammatory molecules by macrophages is a critical component of the acute phase response and has the potential to affect the adaptive immune response that subsequently develops.

IL-10 has been associated with the inhibition of Th1-type immune responses. IL-10 has been shown to inhibit the production of Th1 cytokines and to decrease the proliferation of Th1 cells to antigen (5, 6). The administration of exogenous IL-10 can diminish the toxicity of LPS (7). IL-10 has macrophage-deactivating effects and can inhibit the production of IL-12 by macrophages (8, 9). It is now well established that IL-12 plays an important role in the development of Th1-type immune responses (2). This cytokine is a potent inducer of IFN-γ from T and NK cells, and has been shown to play a crucial role in the development of immunity to intracellular pathogens (10, 11).

In this study, we examine the production of IL-10 and IL-12 by macrophages and the influence that phagocytic receptor ligation can exert on this production. We demonstrate that the ligation of FcγRI can enhance the production of IL-10, reversing the proinflammatory response of macrophages to stimuli such as bacteria or bacterial products.

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Materials and Methods

Mice and Macrophages. 6–8-wk-old BALB/c and C57BL/6 mice were obtained from Taconic Farms, Inc. (Germantown, NY). FcR γ chain–deficient ($FcR\gamma^{-/-}$) and $Fc\gamma RII^{-/-}$ mice (12, 13) were provided by Dr. Jeffrey Ravetch (The Rockefeller University, New York). $Fc\gamma RIII^{-/-}$ mice (14) were provided by Dr. J. Sjef Verbeek (University Hospital Utrecht, Utrecht, The Netherlands). Bone marrow–derived macrophages (BMMϕ) were established as described previously (15).

Opsonized Erythrocytes. IgG-opsonized sheep erythrocytes (E-IgG) were generated by incubating SRBC (Lampire Biological Laboratories, Pipersville, PA) with rabbit anti-SRBC IgG (Organon Teknika-Cappel, Durham, NC) at nonagglutinating titers for 40 min at room temperature. E-IgG were washed and resuspended in HBSS (GIBCO BRL, Gaithersburg, MD) before their addition to macrophages. Complement-opsonized erythrocytes (E-C3bi) were generated by incubating SRBC with culture supernatants of hybridoma S-S.3 (anti-SRBC IgM/κ; American Type Culture Collection, Rockville, MD) at nonagglutinating titers for 40 min at room temperature. IgM-opsonized erythrocytes were washed twice with HBSS and resuspended at 10^8 cells/ml in HBSS with 10% murine C5–deficient serum. After a 15-min incubation at 37°C, E-C3bi were washed and resuspended in HBSS before their addition to macrophages. Erythrocytes were added to macrophage monolayers at a ratio of 20:1.

Macrophage Stimulation. BMMϕ monolayers were stimulated with LPS (*Escherichia coli* 0127:B8; Sigma Chemical Co., St. Louis, MO) at a final concentration of 100 ng/ml, in the presence or absence of opsonized erythrocytes. Cytokine levels in cell supernatants were measured by ELISA 24 h after the addition of stimuli. For mRNA analysis, cells were harvested 6 h after the addition of stimuli, and cytokine mRNA levels were determined by reverse transcription (RT)-PCR, as described previously (15). In some instances, macrophages were stimulated with heat-killed bacteria. The *Eagan* clinical isolate of type b *Haemophilus influenzae* has been described and characterized previously (16). Organisms were grown for 3 h at 37°C in brain–heart infusion broth (Difco Laboratories Inc., Detroit, MI) supplemented with NAD and hemin and then washed twice in HBSS. Bacteria were heat killed by incubating at 60°C for 15 min. Bacteria were opsonized by incubation with anti–*H. influenzae* polyserotype antiserum (Difco Laboratories Inc.) at a 1:25 dilution for 15 min at room temperature. IgG-opsonized or unopsonized bacteria were added to monolayers of BMMϕ, at a ratio of 130 bacteria per macrophage. Cytokine levels in cell supernatants were measured by ELISA 24 h after the addition of bacteria. In some studies, cytokine production induced by LPS or IgG-LPS was examined. IgG-LPS was generated by incubating LPS (*E. coli* 0128:B12, 100 μg/ml; Sigma Chemical Co.) with rabbit anti-LPS polyclonal antiserum (Calbiochem-Novabiochem, San Diego, CA) at a 1:1 dilution for 15 min at 4°C. For in vitro studies, LPS or IgG-LPS was added to monolayers of BMMϕ at a final LPS concentration of 100 ng/ml. For in vivo challenge studies, recombinase-activating gene (RAG)-$1^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, ME) received either IgG-LPS or LPS intravenously (tail vein) at a final LPS dose of 4 μg per mouse. Control LPS was incubated with an equal volume of HBSS. Mice were bled by retroorbital puncture at the indicated time intervals, and serum cytokine levels were determined by ELISA.

Cytokine ELISAs. Levels of murine cytokines were measured by ELISA using appropriately diluted culture supernatants or serum. IL-10 concentrations were determined with a mouse IL-10 ELISA kit (Genzyme Corp., Cambridge, MA, or Biosource International, Camarillo, CA) according to the manufacturer's instructions. Murine IL-12(p40) levels were measured with a mouse IL-12 ELISA kit (Biosource International) according to the manufacturer's instructions. Murine IL-12(p70) levels were measured by ELISA using mAbs C18.2 (anti–murine IL-12 p35) and C17.15 (biotinylated anti–murine IL-12 p40) as ELISA capture and detection antibodies, respectively, according to protocols provided by PharMingen (San Diego, CA). Recombinant murine IL-12 (Genzyme Corp.) was used as a standard. mAbs C18.2 and C17.15 were purified from ascitic fluid provided by Dr. Giorgio Trinchieri (The Wistar Institute, Philadelphia, PA).

Results

Effect of FcγR Ligation on Macrophage IL-10 Production. The production of IL-10 by BMMϕ was examined after specific receptor ligation. BMMϕ were stimulated either with LPS alone, or with LPS in the presence of erythrocytes opsonized with either IgG or complement. The addition of LPS to monolayers of BMMϕ induced a modest but significant production of IL-10 by macrophages. However, the ligation of FcγR simultaneously with the addition of LPS enhanced markedly the production of IL-10. This enhancement was observed at both the mRNA (Fig. 1 *A*) and protein (Fig. 1 *C*) levels. IL-10 mRNA was increased by four- to eightfold (Fig. 1 *B*), and protein secretion was increased by greater than sixfold after FcγR ligation (Fig. 1 *C*). The induction of IL-10 was specific to the FcγR, because ligation of macrophage complement receptors did not significantly alter IL-10 mRNA (Fig. 1 *A*) or protein (Fig. 1 *C*) production. The ligation of macrophage FcγR or complement receptors in the absence of LPS was not sufficient to induce the production of notable levels of IL-10 (Fig. 1 *C*, *inset*).

Effect of FcγR Ligation on IL-10 Production in Macrophages from Gene Knockout Mice. To determine the FcγR subtype responsible for IL-10 upregulation, BMMϕ from gene knockout mice were studied. The FcR γ chain is an essential component of both the FcγRI and FcγRIII, and is required for both receptor assembly and signaling (12). Macrophages from mice lacking the common γ chain ($FcR\gamma^{-/-}$) failed to upregulate IL-10 production in response to E-IgG (Fig. 2), implicating one of these two receptors in this phenomenon. Macrophages derived from mice lacking either the FcγRII or the FcγRIII were fully capable of upregulating IL-10 production in response to E-IgG (Fig. 2). These results are consistent with the high affinity FcγRI being the mediator of IL-10 induction.

Macrophage-derived IL-10 Can Suppress the Production of IL-12. Studies were undertaken to determine whether the amount of IL-10 produced by macrophages in response to FcγR ligation was adequate to suppress IL-12 production. Macrophages were stimulated with LPS in the presence of FcγR ligation for 24 h. Supernatants from these monolayers were collected and assayed for their ability to inhibit IL-12 production. Monolayers of BMMϕ were primed with IFN-γ and then stimulated with LPS in the

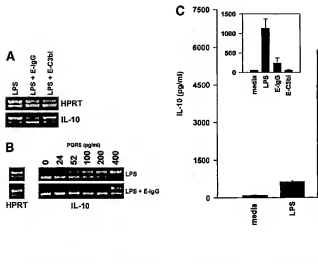

Figure 1. FcγR ligation enhances LPS-induced IL-10 production. (*A*) BMMϕ were exposed to either LPS alone or LPS in combination with either E-IgG or E-C3bi. 6 h after the addition of stimuli, total RNA was isolated and used to carry out competitive RT-PCR. Input cDNAs were adjusted to yield comparable ratios of competitor (*upper band* in each reaction) to wild-type (*lower band* in each reaction) intensities for the amplification reaction for hypoxanthine-guanine phosphoribosyltransferase (HPRT), as resolved on a 2% ethidium-stained agarose gel. The adjusted input cDNAs were then used in subsequent RT-PCR reactions using primers for IL-10. Results are representative of two separate experiments. (*B*) cDNA generated from BMMϕ exposed to LPS or LPS in combination with E-IgG, were first normalized for HPRT levels. Constant volumes of normalized cDNAs were then amplified in the presence of increasing concentrations of competitor (*PQRS*), using primers for IL-10. The concentration of the experimental cDNA is represented by the equivalent intensities of competitor and wild-type bands. The fold increase in IL-10 levels between BMMϕ exposed to LPS or LPS in combination with E-IgG can be determined by taking the ratio of their equivalence points. (*C*) BMMϕ were exposed to either media, LPS, E-IgG, or E-C3bi (*inset*), or LPS alone or LPS in combination with either E-IgG or E-C3bi. After 24 h, the supernatant was harvested, and IL-10 levels were determined by ELISA. Values represent the mean of three independent experiments, each performed in triplicate, ±SE.

presence or absence of a 33% supernatant from LPS/FcγR-stimulated macrophages. 24 h after this stimulation, the production of IL-12(p70) was measured by ELISA. The supernatants from LPS/FcγR-stimulated BMMϕ reduced IL-12(p70) secretion to background levels (Fig. 3). Treating these inhibitory supernatants with a neutralizing mAb to IL-10 partially restored IL-12(p70) production. These results indicate that the IL-10 produced by macrophages after LPS/FcγR stimulation is adequate to inhibit the production of IL-12 by IFN-γ–primed macrophages.

Modulating Macrophage Proinflammatory Responses by Ligating FcγR. Cytokine production by macrophages in response to potential proinflammatory stimuli was examined after FcγR ligation. IL-10 and IL-12(p40) levels were measured by ELISA 24 h after the addition of either LPS or IgG-opsonized LPS to BMMϕ. As expected, LPS induced a potent proinflammatory response by macrophages, characterized by moderate levels of IL-10 (Fig. 4 *A*) and high levels of IL-12(p40) (Fig. 4 *B*). In contrast to this, IgG-opsonized LPS induced higher levels of IL-10 and only modest levels of IL-12(p40). Similar studies were performed using the Gram-negative bacterium, *H. influenzae*. Cytokine production by macrophages in response to unopsonized heat-killed type b *H. influenzae* was compared with that induced in response to IgG-opsonized heat-killed bac-

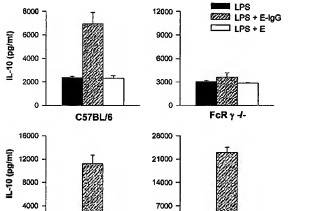

Figure 2. FcγRI is responsible for the FcγR-mediated enhancement of IL-10 production. BMMϕ from C57BL/6, FcRγ$^{-/-}$, FcγRII$^{-/-}$, or FcγRIII$^{-/-}$ mice were exposed to LPS alone or LPS in combination with either E-IgG or unopsonized erythrocytes (*E*). After 24 h, the supernatant was harvested, and IL-10 levels were determined by ELISA. Determinations were performed in triplicate, and values are expressed as the means ± SD. Results are representative of three separate experiments.

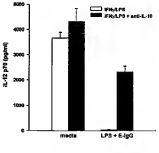

Figure 3. IL-10 produced by macrophages stimulated with LPS/FcγR can suppress IL-12 production. Supernatants from BMMϕ exposed to either media alone or LPS in combination with E-IgG for 24 h were harvested and filtered through a 0.2-μm filter. Supernatants were diluted 1:3 with media and incubated for 15 min at 4°C in either the presence or absence of a neutralizing mAb to IL-10 (JESS-2A5; 20 μg/ml). Diluted supernatants were then added to BMMϕ that had been primed with IFN-γ (100 U/ml) for 8 h, and immediately treated with LPS. After 24 h, the supernatant was harvested, and IL-12(p70) levels were determined by ELISA. Values represent the mean of three independent experiments, each performed in triplicate, ±SE.

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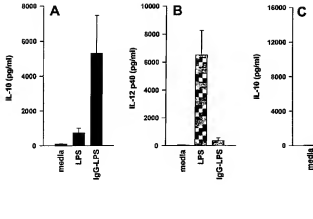

Figure 4. The modulation of inflammatory response by FcγR ligation. BMMϕ were exposed to either media, LPS, or IgG-LPS (*A* and *B*). After 24 h, the supernatant was harvested, and IL-10 (*A*) and IL-12(p40) (*B*) levels were determined by ELISA. Determinations were performed in triplicate, and values are expressed as the means ± SD. Results are representative of four separate experiments. BMMϕ were incubated with media alone or with equal numbers of either unopsonized or IgG-opsonized *H. influenzae* (*C* and *D*). After 24 h, the supernatant was harvested, and IL-10 (*C*) and IL-12(p40) (*D*) levels were determined by ELISA. Determinations were performed in triplicate, and values are expressed as the means ± SD. Results are representative of three separate experiments.

teria. Unopsonized *H. influenzae* induced the production of relatively high levels of both IL-10 (Fig. 4 *C*) and IL-12(p40) (Fig. 4 *D*). However, IgG-opsonized bacteria induced a significant decrease in the production of IL-12(p40) protein and an increase in the production of IL-10. Thus, in both in vitro models, the ligation of FcγR by opsonization with IgG resulted in a reduction in macrophage proinflammatory responses.

Modulation of In Vivo Responses to Bacterial Endotoxin. Studies similar to the in vitro studies performed above were repeated in experimental animals. Several groups have demonstrated that the administration of LPS to experimental animals results in the rapid production of proinflammatory cytokines (17). Given our in vitro observations, we sought to determine whether IgG opsonization of LPS could reverse the inflammatory cytokine response to LPS in vivo. These studies were performed in RAG-$1^{-/-}$ mice, since recent studies have demonstrated that normal mice have naturally occurring antibodies to LPS (18). Mice were injected with either LPS or IgG-LPS, and the generation of cytokines in serum was analyzed over the ensuing 24 h. The injection of low levels (4 μg) of LPS into RAG-$1^{-/-}$ mice induced the transient production of relatively high levels of serum IL-12(p40) (Fig. 5 *A*) and only modest levels of IL-10 (Fig. 5 *B*). The observation that RAG-$1^{-/-}$ mice make high amounts of IL-12 in response to low levels of LPS is consistent with previous observations that antibody-deficient mice are hypersusceptible to LPS (18). The injection of IgG-opsonized LPS into these mice induced an alteration in the cytokine profile. RAG-$1^{-/-}$ mice injected with IgG-LPS made only modest levels of IL-12(p40) (Fig. 5 *A*), but they more than doubled their production of IL-10 (Fig. 5 *B*). This reciprocal alteration in the pattern of cytokine production suggests that IgG opsonization of LPS not only increases the rate of LPS clearance through FcγR, but in doing so also mediates a desirable effect by dampening the proinflammatory response to LPS.

Discussion

Monocytes and macrophages are a primary source of IL-12. IL-12 is a potent inducer of cell-mediated immune responses, and animals lacking IL-12 are invariably more susceptible to infections with intracellular pathogens (2). Because IL-12 plays such a central role in the development of Th1-type immune responses, we have begun to examine the regulation of IL-12 production in macrophages. We have described previously a mechanism whereby receptor ligation can downmodulate IL-12 production by macro-

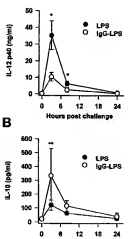

Figure 5. Production of IL-12(p40) and IL-10 in a murine model of septic shock. RAG-$1^{-/-}$ mice received either LPS or IgG-LPS intravenously at a final LPS dose of 4 μg per mouse. Serum levels of IL-12(p40) (*A*) and IL-10 (*B*) were measured at the indicated times after challenge. Data show the mean ± SD of groups of four separately handled mice. *$P < 0.01$, and **$P < 0.08$ (significant by rank-sum analysis) versus the LPS-treated group as determined by Student's *t* test.

phages (15). In this work, we describe a second novel mechanism of downregulating IL-12. This mechanism is distinct from the previously described mechanism in several important ways. First, the present mechanism is not a direct regulation of IL-12 transcription, but rather depends on the production of the inhibitory cytokine IL-10. Second, this regulation is specific to a single receptor class on macrophages, the FcγRI. We show that ligating the macrophage FcγRI increases IL-10 mRNA, resulting in a substantial increase in IL-10 secretion. This macrophage-derived IL-10 is a potent inhibitor of IL-12 production by macrophages. Even IFN-γ–primed macrophages fail to make IL-12 in response to LPS when exposed to macrophage supernatants containing IL-10. Thus, the ligation of the macrophage FcγRI can downmodulate IL-12 production via a mechanism that is dependent on macrophage-derived IL-10.

In identifying the FcγRI as the macrophage receptor that upregulates IL-10 production, we can now associate distinct biological activities with each of the three FcγR classes. CD16, the FcγRIII, is the prototypical proinflammatory Fcγ receptor. Ligating FcγRIII has been associated with the production of proinflammatory cytokines (19), and mice lacking FcγRIII undergo diminished Arthus reactions (14). CD32, the FcγRII, is a negative regulator of immune complex–triggered immune responses, and mice lacking FcγRII have augmented anaphylactic responses to IgG (13). Our studies would classify the FcγRI (CD64) as another inhibitory FcγR, but by a different mechanism than that observed for FcγRII. Whereas FcγRII inhibits signaling (20), FcγRI actively promotes the transcription of an inhibitory cytokine, IL-10. Thus, by two distinct mechanisms, both the FcγRI and FcγRII can inhibit inflammatory responses to immune complexes. Previous observations that immune complexes can inhibit both the in vivo clearance of *Listeria monocytogenes* (21) and the in vitro macrophage tumoricidal and cytotoxic activity (22, 23) are consistent with FcγR ligation leading to an inhibition of immune responses.

The in vitro studies presented here indicate that FcγRI ligation has the potential to dampen the acute response to inflammatory stimuli such as LPS or Gram-negative bacteria. In both cases, opsonization with IgG increased macrophage IL-10 production and diminished IL-12 production. The prediction from these studies is that bacterial clearance in an immune animal may be associated with a diminished inflammatory response relative to nonimmune animals. Furthermore, targeting LPS specifically to FcγRI might be a practical way of eliminating endotoxin without the consequent proinflammatory sequelae. The reciprocal alteration of IL-10 and IL-12 after FcγR ligation also has the potential to exert an impact on the acquired immune response, biasing it towards a Th2-type response. The implication from these studies is that IgG itself may be an important promoter of the Th2-type immune response.

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References

1. Nathan, C.F. 1987. Secretory products of macrophages. *J. Clin. Invest.* 79:319–322.
2. Trinchieri, G., and F. Gerosa. 1996. Immunoregulation by interleukin-12. *J. Leukocyte Biol.* 59:505–511.
3. Dalrymple, S.A., L.A. Lucian, R. Slattery, T. McNeil, D.M. Aud, S. Fuchino, F. Lee, and R. Murray. 1995. Interleukin-6-deficient mice are highly susceptible to *Listeria monocytogenes* infection: correlation with inefficient neutrophilia. *Infect. Immun.* 63:2262–2268.
4. Kincy-Cain, T., J.D. Clements, and K.L. Bost. 1996. Endogenous and exogenous interleukin-12 augment the protective immune response in mice orally challenged with *Salmonella dublin*. *Infect. Immun.* 64:1437–1440.
5. Fiorentino, D.F., A. Zlotnik, P. Vieira, T.R. Mosmann, M. Howard, K.W. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol.* 146:3444–3451.
6. de Waal Malefyt, R., J. Haanen, H. Spits, M.G. Roncarolo, A. te Velde, C. Figdor, K. Johnson, R. Kastelein, H. Yssel, and J.E. de Vries. 1991. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J. Exp. Med.* 174:915–924.
7. Howard, M., T. Muchamuel, S. Andrade, and S. Menon. 1993. Interleukin 10 protects mice from lethal endotoxemia. *J. Exp. Med.* 177:1205–1208.
8. Bogdan, C., Y. Vodovotz, and C. Nathan. 1991. Macrophage

deactivation by interleukin 10. *J. Exp. Med.* 174:1549–1555.
9. D'Andrea, A., M. Aste-Amezaga, N.M. Valiante, X. Ma, M. Kubin, and G. Trinchieri. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte IFN-γ production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J. Exp. Med.* 178:1041–1048.
10. Heinzel, F.P., D.S. Schoenhaut, R.M. Rerko, L.E. Rosser, and M.K. Gately. 1993. Recombinant interleukin 12 cures mice infected with *Leishmania major*. *J. Exp. Med.* 177:1505–1512.
11. Tripp, C.S., W.F. Wolf, and E.R. Unanue. 1993. Interleukin-12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis and interleukin-10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. USA.* 90:3725–3729.
12. Takai, T., M. Li, D. Sylvestre, R. Clynes, and J.V. Ravetch. 1994. FcR γ chain deletion results in pleiotropic effector cell defects. *Cell.* 76:519–529.
13. Takai, T., M. Ono, M. Hikida, H. Ohmori, and J.V. Ravetch. 1996. Augmented humoral and anaphylactic responses in FcγRII-deficient mice. *Nature.* 379:346–349.
14. Hazenbos, W.L., J.E. Gessner, F.M. Hofhuis, H. Kuipers, D. Meyer, I.A. Heijnen, R.E. Schmidt, M. Sandor, P.J. Capel, M. Daeron, et al. 1996. Impaired IgG-dependent anaphylaxis and Arthus reaction in FcγRIII(CD16) deficient mice. *Immunity.* 5:181–188.
15. Sutterwala, F.S., G.J. Noel, R. Clynes, and D.M. Mosser. 1997. Selective suppression of interleukin-12 induction after macrophage receptor ligation. *J. Exp. Med.* 185:1977–1985.
16. Noel, G.J., S.K. Hoiseth, and P.J. Edelson. 1992. Type b capsule inhibits ingestion of *Haemophilus influenzae* by murine macrophages: studies with isogenic encapsulated and unencapsulated strains. *J. Infect. Dis.* 166:178–182.
17. Wysocka, M., M. Marek, L.Q. Vieira, L. Ozmen, G. Garotta, P. Scott, and G. Trinchieri. 1995. Interleukin-12 is required for interferon-γ production and lethality in lipopolysaccharide-induced shock in mice. *Eur. J. Immunol.* 25: 672–676.
18. Reid, R.R., A.P. Prodeus, W. Khan, T. Hsu, F.S. Rosen, and M.C. Carroll. 1997. Endotoxin shock in antibody-deficient mice: unraveling the role of natural antibody and complement in the clearance of lipopolysaccharide. *J. Immunol.* 159:970–975.
19. Cassatella, M.A., I. Anegón, M.C. Cuturi, P. Griskey, G. Trinchieri, and B. Perussia. 1989. FcγR(CD16) interaction with ligand induces Ca^{2+} mobilization and phosphoinositide turnover in human natural killer cells: role of Ca^{2+} and FcγR(CD16)-induced transcription and expression of lymphokine genes. *J. Exp. Med.* 169:549–567.
20. Muta, T., T. Kurosaki, Z. Misulovin, M. Sanchez, M.C. Nussenzweig, and J.V. Ravetch. 1994. A 13 amino acid motif in the cytoplasmic domain of FcγRIIB modulates B cell receptor signaling. *Nature.* 368:70–73.
21. Tripp, C.S., K.P. Beckerman, and E.R. Unanue. 1995. Immune complexes inhibit antimicrobial responses through interleukin-10 production: effects in severe combined immunodeficient mice during *Listeria* infection. *J. Clin. Invest.* 95: 1628–1634.
22. Esparza, I., R. Green, and R.D. Schreiber. 1983. Inhibition of macrophage tumoricidal activity by immune complexes and altered erythrocytes. *J. Immunol.* 131:2117–2121.
23. Virgin, H.W., E.A. Kurt-Jones, G.F. Wittenberg, and E.R. Unanue. 1985. Immune complex effects on murine macrophages. II. Immune complex effects on activated macrophages cytotoxicity, membrane IL-1, and antigen presentation. *J. Immunol.* 135:3744–3749.

STUDIES OF AGGREGATED γ-GLOBULIN

I. Sedimentation, Electrophoretic and Anticomplementary Properties

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Since the demonstrations by Bordet and Gengou that immune reactions inactivate serum complement (C′), this principle has been applied in the development of many serodiagnostic tests (1). Although, in general, C′ is not fixed by either antigen or immune serum alone, a small percentage of heat inactivated (56°C, 30 min) human sera demonstrate anticomplementary activity. Works of Jersild (2) and Nørgaard (3, 4) have related anticomplementary activity of these sera to both the degree of hypergammaglobulinemia and the extent to which sera were heated. Davis *et al.* demonstrated that normal γ-globulin isolated by electrophoresis inactivated C′, but that reconstitution with other serum fractions prevented anticomplementary activity (5).

The observations in the present report derive from a study of certain properties of rheumatoid arthritis sera. In the majority of such sera, a macromolecular complex is formed between a γ-globulin of the 19S molecular class ("rheumatoid factor") and several moles of 7S γ-globulin (6). This complex, which has a sedimentation constant of approximately 22, reacts in a variety of serologic systems, *i.e.*, sensitized sheep cell agglutination (7), agglutination of Rh sensitized human erythrocytes (8, 9) and absorption onto immune complexes (10). In addition to the above systems, all of which utilize as the basis for sensitization an immune reaction, there is another class of rheumatoid serologic tests which is based on a reaction between the "rheumatoid factor" and some preparations of pooled human γ-globulin, *i.e.*, F II sheep cell agglutination (11), F II precipitin reaction (12), and F II latex fixation tests (13). Recent studies of the F II sheep cell agglutination and F II precipitin reactions indicate that the reactive material in Cohn fraction II γ-globulin (F II) consists of artificially formed molecular aggregates of 7S γ-globulin (6, 14). It was suggested that the requirement of aggregation in this group of nonimmune sensitized systems (F II reactions) might be simulating the aggregation of antibody γ-globulin that occurred in the immune sensitized systems (14). In the course of complement fixation studies, it was noted that aggregated γ-globulin was intensely anticomplementary.

Evidence will be presented which a) confirms the observation that γ-globulin by itself is anticomplementary, and b) demonstrates that the magnitude of C′ destruction correlates with the degree to which γ-globulin is aggregated. Preliminary studies suggest that the reaction of aggregated γ-globulin with C′ results in individual component inactivation that resembles the inactivation of C′ by immune reactions.

MATERIALS AND METHODS

Commercial pooled human Cohn fraction II (E. R. Squibb & Sons) constituted the source of γ-globulin used in the present study.

Concentration of aggregated γ-globulin was accomplished by fractionating a solution of F II which had been heated to 56°C for 30 min with sodium sulfate (14). Fractions SS_1, SS_1, SS_2, SS_4, SS_4 and SS_8 were precipitated at molarities of sodium sulfate 0.36, 0.62, 0.81, 0.96, 1.08 and 1.18, respectively. Fractions SS_1 and SS_2 contained aggregates of γ-globulin, and fractions SS_2 to SS_8 were devoid of detectable aggregated material.

Solid γ-globulin was prepared by repeated saline washing of saline-insoluble material that formed during the process of heating solutions of F II.

Electrophoretic studies were performed by the moving boundary technique in a Perkin-Elmer model 38 instrument. Electrophoretic mobilities were determined using veronal buffer, pH 8.6,

ionic strength 0.1,nd a expressed as distance (cm) $\times 10^{-5}$ cm^2 volt^{-1} sec.$^{-1}$.

Sedimentation studies were performed in a Spinco model E analytical ultracentrifuge.

Nitrogen determinations were by the micro Kjeldahl technique (Markham) (15).

Estimation of hemolytic activity of C′ was performed by the method of Mayer *et al.* (16), and expressed as 50% hemolytic units ($C'H_{50}$). Pools of normal guinea pig sera varied between 200 and 250 $C'H_{50}$ units. Complement reagents (treatment of guinea pig sera with heat, Zymosan and ammonia) were prepared as described by Kabat and Mayer (17). Immune decomplementation was accomplished by absorbing 1 ml of guinea pig pool with 0.1 mg nitrogen of washed immune precipitate (bovine serum albumin (BSA)-rabbit anti-BSA formed at equivalence) at 0–4°C for 24 hr. Incubation of 1 ml of guinea pig sera pool with 0.40 mg of solid γ-globulin nitrogen at 0–4°C for 24 hr resulted in inactivation of C′ to the extent that 1 ml of a 1:10 dilution of the supernatant gave no hemolysis.

Anticomplementary studies were conducted by incubating known amounts of guinea pig C′ with varying amounts of test materials at 0–4°C for 18 hr, with subsequent determination of $C'H_{50}$ levels.

Reconstitution experiments with various C′ depleted reagents were performed by mixing 1-ml aliquots of paired reagents diluted 1:10. Experiments were performed in triplicate so that both qualitative and quantitative determinations of hemolytic activity could be determined, i.e., paired reagents which gave complete

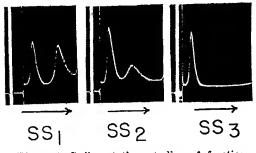

Figure 1. Sedimentation studies of fractions SS_1, SS_2 and SS_3 of Cohn Fraction II. Photographs are exposures after 32 min at 47660 rpm. Direction of sedimentation noted. The slower sedimenting component in each fraction had a sedimentation constant of approximately 7.

TABLE I

*Electrophoretic mobilities of sodium sulfate fractions of Cohn F II**

Fractions	Electrophoretic Mobility of Main Component†
SS_1	1.89
SS_2	1.84
SS_3	1.29
SS_4	1.30
SS_5 + SS_6	1.40

* Heated at 56°C for 30 min before Fractionation.
† Measurements from the ascending sides.

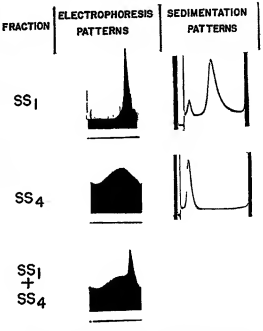

Figure 2. Electrophoretic and sedimentation patterns of fractions SS_1 and SS_4. The directions of sedimentation and electrophoresis are to the right. Electrophoresis photographs are of ascending limbs after 210 min (see Materials and Methods). Sedimentation patterns of SS_1 and SS_4 were exposures after 16 and 32 min, respectively, at 47660 rpm.

The nitrogen concentrations of SS_1 and SS_4 in the electrophoretic studies were 3.4 and 4.1 mg/ml, respectively. The minor 7S component in the sedimentation study of SS_1 represented 8% of the total fraction (estimated by planimetry with correction for the sector-shaped centrifuge cell).

hemolysis were diluted and different aliquots taken for $C'H_{50}$ levels.

RESULTS

Sedimentation studies. Figure 1 illustrates sedimentation patterns of fractions SS_1, SS_2 and SS_3. Fractions SS_1 and SS_2 demonstrate components with sedimentation constants greater than 7. In the present study, absolute S_{20} values were not determined. In previous experiments, the sedimentation constants for the heavy components in fractions SS_1 and SS_2 were 40 and 30, respectively, (14). It should be emphasized that aggregates are polydispersed, and that there is no evidence that such materials are present as such in unaltered sera. There is every indication that aggregation occurs in the process of commercial fractionation of sera or as a result of purposeful denaturation, *i.e.*, heat in the present study. In the author's experience, electrophoretically isolated γ-globulin does not contain detectable aggregated material.

TABLE II

*Comparison of anticomplementary properties of Cohn F II and sodium sulfate fractions of Cohn F II**

Fraction	Minimum Quantity of Nitrogen Which Reduced 16 $C'H_{50}$ Units to Less than 1 Unit
	mg
Cohn F II	0.300
SS_1	0.011
SS_2	0.028
SS_3	1.90
SS_4	24.2

* Incubations were 18 hr at 0–4°C, each tube containing guinea pig serum diluted 1:10. In the control tube, containing only serum, 16 $C'H_{50}$ units remained after 18 hr.

Electrophoretic studies. Table I summarizes electrophoretic mobility data. In several determinations, the main components of fractions SS_1 and SS_2 migrated slightly faster than the fractions that did not contain aggregates (SS_3, SS_4, and SS_2-SS_3), and formed sharper boundaries than nonaggregated γ-globulin. Minor components in some preparations of SS_1 and SS_2 had mobilities in the range of 1.0 to 1.3. Electrophoretic and sedimentation studies of SS_1 and SS_4 are shown in Figure 2. When fractions SS_1 and SS_2 were mixed, the sharp electrophoretic boundary formed by the aggregated fraction migrates faster than the peak of the broad component in fraction SS_4. The small 7S component in the sedimentation pattern of this particular preparation of SS_1 represented 8% of the total

TABLE III

Complement studies on mixing of guinea pig serum reagents

Treatment of Guinea Pig Serum*	Expected C′ Component Deficit	Tube Numbers									
		1	2	3	4	5	6	7	8	9	10
Absorbed with solid γ-globulin	?	1 ml	1 ml	1 ml	1 ml						
Heated 56°C 30 min.	C′ 1 and 2	1 ml				1 ml	1 ml	1 ml			
Absorbed with Zymosan	C′ 3		1 ml			1 ml			1 ml	1 ml	
Ammonia-treated	C′ 4			1 ml			1 ml		1 ml		1 ml
Absorbed with antigen-antibody	C′ 1, 2 and 4				1 ml			1 ml		1 ml	1 ml
O.D. 550		0.070	0.550	0.500	0.040	0.560	0.560	0.040	0.555	0.560	0.510
Hemolysis (%)		<10	100	90	<10	100	100	<10	100	100	90
Quantitative estimation of C′ 50% units		<1	3.5	2	<1	12	20	<1	10	4	2

* All guinea pig serum reagents were diluted 1:10 after treatment. One-milliliter aliquots alone showed no hemolysis and were not anticomplementary when tested with 2 $C'H_{50}$ units of guinea pig pool.

fraction, an amount insufficient for resolution in the electrophoretic study.

Anticomplementary studies. Table II compares the relative anticomplementary properties of five different fractions of γ-globulin. Fraction SS_4 demonstrates inactivation of 16 $C'H_{50}$ units at a nitrogen level of 24.2 mg, which was over 2000 times the amount of fraction SS_1 required for comparable C′ inactivation.

Reconstitution of C′ reagents. Table III summarizes an experiment in which paired 1.0-ml aliquots of five reagents were mixed and resultant $C'H_{50}$ levels determined. As outlined in Materials and Methods, quantitative $C'H_{50}$ determinations were performed on paired reagents which demonstrated complete hemolysis. Guinea pig serum absorbed with solid γ-globulin demonstrated some repletion of hemolytic activity when added to Zymosan and ammonia-treated reagents, but no hemolytic activity when added to guinea pig serum decomplemented by heat or absorption with immune precipitate. Comparisons of $C'H_{50}$ levels of paired reagents is summarized in Table IV. The only two reagents which give equivalent hemolytic activity when added to the other three reagents were the immune precipitate and solid γ-globulin absorbed sera.

Results similar to the above were obtained in experiments utilizing guinea pig serum depleted of hemolytically active C′ by the addition of soluble aggregated γ-globulin, *i.e.*, fraction SS_2 instead of solid γ-globulin. The latter method was favored, since it avoided the risk of making the depleted reagent anticomplementary.

TABLE IV

*Comparisons of hemolytic activity ($C'H_{50}$ units) of paired complement reagents**

	Immune Precipitate Absorbed	Solid γ-Globulin Absorbed	Heated 56°C	Ammonia-Treated	Zymosan-Absorbed
Immune precipitate absorbed...		<1	<1	2	4
Solid γ-globulin absorbed...	<1		<1	2	3.5
Heated 56°C.	<1	<1		20	12
Ammonia-treated....	2	2	20		10
Zymosan-absorbed...	4	3.5	12	10	

* Summary of data in Table III.

DISCUSSION

The phenomena herein reported, *i.e.*, aggregation of γ-globulin with heat and subsequent inactivation of C′, are probably the basis for anticomplementary properties of some human sera. Complement inactivation of such sera is related to the concentration of γ-globulin and the extent to which sera are heated (2, 3, 4). (Maximum anticomplementary effect is obtained at temperatures between 52° and 62°C. Sera decomplemented by means other than heat are not anticomplementary.) Sera of patients with multiple myeloma comprise the majority of these sera; indeed, the finding of anticomplementary properties has been cited as presumptive evidence of myelomatosis (18). Bloom *et al.* have described a case in which anticomplementary activity of heated sera preceded the clinical onset of multiple myeloma by 17 years (19). Nørgaard has offered indirect evidence that the destruction of C′ by heated hypergammaglobulinemic sera is related to the formation of molecular aggregates (20) and that the specific component of C′ that is inactivated may be the second component (C'_2) (21). The C′ studies in the present report, although subject to limitations, suggest that more than C'_2 is inactivated by aggregated γ-globulin. (The preparation of C′ reagents conformed to current practice but there is general doubt as to the absolute specificity of these methods.) Since ammonia-treated serum (R4) showed good repletion of hemolytic activity when added to Zymosan-treated serum (R3) and heated serum (R1, 2) but negligible repletion when added to solid γ-globulin absorbed serum, C'_4 appears to be lacking in the latter reagent. C'_1, C'_2, or both, are probably inactivated in solid γ-globulin absorbed serum since its combination with heated serum did not result in hemolysis. The relatively poor repletion of hemolytic activity achieved with Zymosan absorbed serum may reflect the limited concentration of C'_4 in guinea pig serum (22). The final interpretation of the limited data suggests that absorption with solid γ-globulin renders guinea pig serum deficient in $C'_{1,\ 4}$; $C'_{1,\ 2,\ 4}$; or $C'_{2,\ 4}$. Marcus recently presented evidence that serum treated with human γ-globulin coated bentonite particles is deficient in C'_1 and C'_4 (23). The similarity

between C′ component destruction by aggregated γ-globulin and immune aggregates offers basis for speculation as to the way in which C′ participates in immune reactions. Heidelberger *et al.*, in a discussion of this, suggested the possibility that antibody γ-globulin might reversibly combine with C′ in the absence of antigen; irreversible binding of C′ being dependent on the aggregation of antibody with its related antigen (24). These authors stated, "It is conceivable that C'_1 would unite with equal firmness with normal γ-globulin were there a means of bringing sufficient number of such molecules into suitable apposition and holding them there." The C′ inactivating properties of physically aggregated γ-globulin would appear, in part, to fulfill this speculation.

The type of chemical bonds responsible for aggregation of γ-globulin by heat is not known. Aggregates once formed are not dissociated by treatment with concentrated urea or acid solutions (pH 3 to 4). Since aggregated γ-globulin demonstrated slightly faster electrophoretic migration than nonaggregated material (Table I), the aggregates presumably carry a different net charge.

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SUMMARY

Molecular aggregation occurs when solutions of γ-globulin are heated to 56°C. Aggregated γ-globulin, when concentrated by salt fractionation, demonstrated marked anticomplementary properties. Preliminary C′ studies suggested that absorption of guinea pig serum with solid γ-globulin resulted in C′ component destruction that resembled C′ inactivation by immune systems.

ADDENDUM

The technique of Mayer and Levine for stepwise addition of C′ components to sensitized cells (J. Immunol., **75**: 435, 443, 1954) demonstrated that guinea pig serum treated with soluble aggregated gamma globulin lysed cells which had reacted with $C'_{1, 4, 2}$ ($EAC'_{1, 4, 2}$) but not with cells which had reacted with $C'_{1, 4}$ ($EAC'_{1, 4}$). This suggests that inactivation of C′ by aggregated gamma globulin involves destruction of C'_1, C'_2 and C'_4.

REFERENCES

1. Bordet, J. and Gengou, O., Ann. Inst. Pasteur, **15**: 289, 1901.
2. Jersild, P. M., Ztschr. klin. Med., **130**: 670, 1936.
3. Nørgaard, O., Acta path. et microbiol. scandinav., **27**: 811, 1950.
4. Nørgaard, O., Acta path. et microbiol. scandinav., **36**: 365, 1955.
5. Davis, B. D., Kabat, E. A., Harris, A. and Moore, D., J. Immunol., **49**: 223, 1944.
6. Franklin, E. C., Kunkel, H. G., Muller-Eberhard, H. J. and Holman, H. R., J. Exper. Med., **105**: 425, 1957.
7. Rose, H. M., Ragan, C., Pearce, E. and Lipman, M. O., Proc. Soc. Exper. Biol. & Med., **68**: 1, 1948.
8. Waller, M. V. and Vaughan, J. H., Proc. Soc. Exper. Biol. & Med., **92**: 198, 1956.
9. Grubbe, R., Acta path. et microbiol. scandinav., **39**: 195, 1956.
10. Vaughan, J. H., J. Immunol., **77**: 181, 1956.
11. Heller, G., Jacobson, A. S., Kolodny, M. H. and Kammerer, W. H., J. Immunol., **72**: 66, 1954.
12. Epstein, W., Johnson, A. and Ragan, C., Proc. Soc. Exper. Biol. & Med., **91**: 235, 1956.
13. Singer, J. M. and Plotz, C. M., Am. J. Med., **21**: 888, 1956.
14. Christian, C. L., J. Exper. Med., **108**: 139, 1958.
15. Markham, R., Biochem. J., **36**: 790, 1942.
16. Fischel, E. E., Pauli, R. H. and Lesh, J., J. Clin. Invest., **28**: 1172, 1949.
17. Kabat, E. A. and Mayer, M. M., *Experimental Immunochemistry*. Charles C Thomas, Springfield, Ill., 1948.
18. Jersild, P. M., J. A. M. A., **113**: 1119, 1939.
19. Bloom, M. L., Shulman, S. and Witebsky, E., Clin. Res., **6**: 206, 1958.
20. Nørgaard, O., Acta path. et microbiol. scandinav., **36**: 71, 1955.
21. Nørgaard, O., Acta path. et microbiol. scandinav., **27**: 865, 1950.
22. Bier, O. G., Leyton, G., Mayer, M. M. and Heidelberger, M., J. Exper. Med., **81**: 449, 1945.
23. Marcus, D., Fed. Proc., **18**: 583, 1959.
24. Heidelberger, M., Weil, A. J. and Treffers, H. P., J. Exper. Med., **73**: 695, 1941.

STUDIES OF AGGREGATED γ-GLOBULIN

II. Effect *in Vivo*

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In Part I of the present investigations, the anticomplementary property of human γ-globulin[1] (HGG) was related to the extent to which molecular aggregation was present (1). Complement (C′) studies suggested that guinea pig serum absorbed with a saline-insoluble preparation of HGG was devoid of hemolytic activity and that the inactivation of C′ components was similar to the inactivation of C′ by immune precipitates.

The present report concerns studies of the action of aggregated HGG *in vivo*. Intracutaneous injection of aggregated HGG in the guinea pig, rat and man produced inflammation. This property was minimal or absent when nonaggregated HGG was injected. Intravenous injection of aggregated HGG in guinea pigs rendered the sera of recipient animals deficient in hemolytically active C′ for a period of several hours.

MATERIALS AND METHODS

Commercial pooled human Cohn fraction II (F II) which was supplied by E. R. Squibb & Sons, constituted the source of HGG. Solutions of F II in isotonic saline were sterilized by passage through a Seitz filter, heated for 30 min in a 56°C water bath, and then fractionated by precipitation with sodium sulfate (1, 2). Precipitated materials were redissolved in isotonic saline containing Merthiolate in a final concentration of 0.01%. Sterility of all preparations was verified by inoculations into liquid thioglycolate media (Difco) enriched with glucose (0.15%). For the purpose of the present study, fractions SS_1 and SS_2 were pooled as a source of aggregated HGG, and fractions SS_2 to SS_4 were combined as a source of HGG that was free of detectable aggregates.

Nitrogen determinations were by the micro-Kjeldahl technique (3).

[1] We are indebted to the Red Cross Blood Program for supplies of γ-globulin.

Serum C′ levels were estimated by the method of Mayer *et al.* and expressed as 50% hemolytic units ($C'H_{50}$ units) (4) (method summarized in Reference 5).

Skin responses. a) Histologic studies were performed in rats and guinea pigs which received intracutaneous injections. At different intervals thereafter, injection sites were incised, fixed in Bouin's solution, imbedded in paraffin, sectioned and stained with hematoxylin and eosin. b) Human volunteers received intracutaneous in-

TABLE I

*Histologic skin reactions resulting from intracutaneous injection of 0.5 mg (nitrogen) of fractions $SS_1 + SS_2$ and SS_2 to SS_4 in guinea pigs**

Material Injected	Time of Biopsy	Animal Number	Degree of Cellular Infiltration	
			Polymorphonuclear	Mononuclear
$SS_1 + SS_2$ (aggregated γ-globulin)	6 hr	1	+++	0
		2	+++	0
	24 hr	3	+++	++
		4	++++	++++
	48 hr	5	++	+
		6	0	0
	5 days	7	++	++
		8	++	++
	7 days	9	0	+
SS_2 to SS_4 (nonaggregated γ-globulin)	6 hr	10	+	0
		11	+	0
	24 hr	12	+	+
		13	0	0
	48 hr	14	0	0
		15	+	+
	5 days	16	0	0
		17	0	0
	7 days	18	0	0

* Volume of injection 0.1 ml.

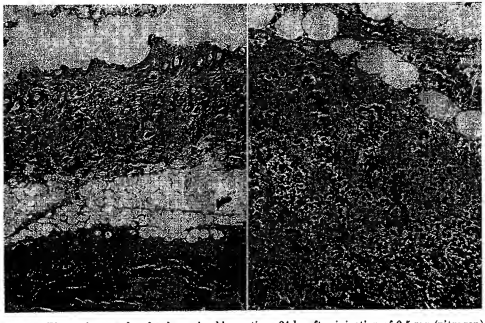

Figure 1. Photomicrographs of guinea pig skin sections 24 hr after injection of 0.5 mg (nitrogen) of fraction $SS_1 + SS_2$. (*A*, *left*) Magnification × 61; (*B*, *right*) magnification × 210.

jections of varying quantities of aggregated HGG ($SS_1 + SS_2$) and nonaggregated HGG (SS_2 to SS_4). Observations were made at intervals regarding swelling, erythema and tenderness. c) Skin reactions were studied in guinea pigs with a modification of the dye technique of Ovary (6). Guinea pigs weighing 200–250 g were given an intravenous injection of 0.5 ml of 1% Evans blue in isotonic saline, followed by intracutaneous injections of varying amounts of test materials. After 30 min, the animals were sacrificed and the injection sites examined for the diameter and intensity of bluing.

RESULTS

Intracutaneous injections of human γ-globulin. Intracutaneous injection of aggregated HGG ($SS_1 + SS_2$) in rats and guinea pigs produced an elevated plaque which appeared after a few hours, persisted several days and was maximal at about 24 hr after injection. Similar injections of nonaggregated HGG (SS_2-SS_4) did not result in gross lesions. Table I summarizes histologic findings in skin biopsies of injection sites in guinea pigs receiving equal quantities of $SS_1 + SS_2$ and SS_2 to SS_4 intracutaneously. Intense polymorphonuclear leukocytic infiltrations were noted in the dermis of $SS_1 + SS_2$ injected sites. This change was present 6 hr after injection, but was maximal in the 24-hr specimens at which time mononuclear cells were abundant. (Photomicrographs of injection sites after 24 hr are shown in Figure 1.)

TABLE II

Skin response in guinea pigs given Evan blue (i.v.) and test materials intracutaneously

Fraction	Quantity (mg N₂) Injected Intracutaneously	Average Diameter (mm) of Bluing 30 min After Injection (Average of Four Animals) *
$SS_1 + SS_2$	0.3	12.5* (range 10–15)
	0.15	11.5* (range 10–12)
	0.075	6.0 (range 5–10)
	0.038	5.0
SS_2 to SS_4	0.3	5.0
	0.15	5.0
	0.075	5.0
	0.038	5.0
Saline		5.0

* Intense blue coloration as opposed to faint bluing in others.

TABLE III

Skin responses in human subjects to intracutaneous injections of fractions $SS_1 + SS_2$ and SS_3 to SS_6 (0.5 mg N_2 in 0.1 ml)

Subject	Material Injected	10 min		30 min		1 hr		2 hr		24 hr	
		Swelling	Erythema	Swelling	Erythema	Swelling	Erythema	Swelling	Erythema	Swelling	Erythema
C. C.	$SS_1 + SS_2$	16*	16	18	18	20	20	35†	35	0†	30 (faint)
	SS_3 to SS_6	8	0	5	0	0	0	0	0	0	0
	Saline	5	0	0	0	0	0	0	0	0	0
C. R.	$SS_1 + SS_2$	12	32	12	32	12	22	10†	10	5†	10 (faint)
	SS_3 to SS_6	5	0	5	0	5	0	0	0	0	0
	Saline	5	0	5	0	5	0	0	0	0	0
R. K.	$SS_1 + SS_2$	6	30	10	30	10	20	6†	6	5†	0
	SS_3 to SS_6	5	0	5	0	5	0	0	0	0	0
	Saline	5	0	5	0	5	0	0	0	0	0

* Numbers indicate the average diameters of reactions in mm.
† Tenderness associated with swelling.

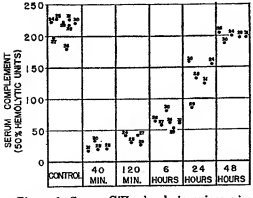

Figure 2. Serum $C'H_{50}$ levels in guinea pigs given aggregated human γ-globulin ($SS_1 + SS_2$ 2.5 mg N_2) intravenously. Numbers of individual animals are indicated.

TABLE IV

Serum $C'H_{50}$ levels at intervals subsequent to intravenous administration of fractions $SS_1 + SS_2$ and SS_3 to SS_6 to guinea pigs

Fraction	N_2 Quantity Injected	Serum Complement ($C'H_{50}$) Levels			
		1 hr	3 hr	6 hr	24 hr
	mg				
$SS_1 + SS_2$	5.0	<5*	10 (range <5–32)	44 (range 30–55)	135 (range 100–150)
SS_3 to SS_6	5.0	140 (range 135–157)	160 (range 144–170)	165 (range 150–175)	170 (range 162–180)

* Figures represent the average of four animals.

Aside from venous and capillary distention, vascular abnormalities were not noted. Slight polymorphonuclear and mononuclear infiltrates were noted in about one-half of the animals injected with nonaggregated HGG (SS_3 to SS_6).

Although immediate skin reactions were not visible grossly, the dye technique (Table II) demonstrated an increase in vascular permeability 30 min after cutaneous injections of $SS_1 + SS_2$. Equivalent amounts of SS_3 to SS_6 did not result in more extravasation of dye than did isotonic saline.

Intracutaneous injections of $SS_1 + SS_2$ into human volunteers produced marked swelling and erythema. These changes, which are summarized in Table III, appeared within 10 min after injection and were not accompanied by wheal reactions. Tenderness was maximal after a few hours but persisted beyond 24 hr. Injections of SS_3 to SS_6 into the skin of the same volunteers did not result in significant changes.

Intravenous injection of HGG. Figure 2 illustrates the changes in serum complement ($C'H_{50}$ units) of guinea pigs receiving 2.5 mg of $SS_1 + SS_2$ nitrogen intravenously. $C'H_{50}$ levels were below 50 units during the first 2 hr and did not return to normal range until 48 hr had elapsed. Although a small percentage of animals demon-

strated anaphylactiklike signs, the majority of them showed no ill effects. Table IV summarizes an experiment in which 5.0 mg (nitrogen) of $SS_1 + SS_2$ and SS_4 to SS_8 were administered intravenously to two groups of guinea pigs. Marked depression of hemolytically active C′ persisted for at least 6 hr in the animals receiving $SS_1 + SS_2$.

DISCUSSION

Certain properties of aggregated HGG ($SS_1 + SS_2$ in the present study) resembled properties of immune complexes: a) inactivation of C′ both *in vitro* and *in vivo*, b) cutaneous reactions (7, 8, 9), and c) reaction with the "rheumatoid factor" (10, 11). Two questions raised by the above are a) "do similar chemical and enzymatic changes mediate the tissue response to both immune aggregates and γ-globulin aggregated by heat?" and b) "what role does C′ play in these responses?" Neither question can be answered at the present time, but a considerable fund of indirect information suggests that C′ is in part responsible for the tissue responses of hypersensitivity (12, 13). In general, the anaphylactogenic property of various animal antisera correlates with the ability of the sera to fix C′ with related antigens. Humphrey and Jaques have demonstrated that a heat labile serum factor and calcium are required for the immune release of histamine from rabbit platelets (14). Immune hemolysis, *i.e.*, lysis of erythrocytes by antibody and C′, has been cited as a model of hypersensitivity (12). Although hemolysin alone does not alter erythrocyte morphology, the addition of small amounts of C′ results in disruption of sensitized cells. It is tempting to relate this type of tissue damage to the enzymatic properties of C′ (esterase activity of activated C′1 (15–18) and proteases associated with C′ action (19–21)).

The lowered serum C′ levels in serum sickness and glomerulonephritis (both human and experimental) give no indication of any pathogenetic role of C′. The most direct evidence of such a role derives from the work of Bier and Osler and others who demonstrated that passive cutaneous anaphylaxis in rats is modified by decomplementation with an unrelated immune system (22, 23). Preliminary experiments of the author have demonstrated that prior injection of a decomplementing dose of $SS_1 + SS_2$ into guinea pigs protects against passive systemic anaphylaxis. These observations have not been sufficiently controlled to justify the conclusion that protection was mediated *via* lowered C′ rather than some other effect of the administered foreign protein.

Acknowledgment. The author is indebted to Mr. Thomas Hayes and Mrs. Gwendolyn Linker for technical assistance and to Dr. Charles Ragan for advice and criticism.

SUMMARY

Injection of aggregated human γ-globulin into the skin of guinea pig, rat and man produced inflammatory changes. Histologically, the injection sites of guinea pigs and rats demonstrated marked polymorphonuclear and mononuclear infiltrations that were maximal at about 24 hr.

Intravenous administration of aggregated human γ-globulin, which is strikingly anticomplementary *in vitro*, rendered the sera of recipient animals deficient in hemolytically active C′ for several hours.

REFERENCES

1. CHRISTIAN, C. L., J. Immunol., 84: 112, 1960.
2. CHRISTIAN, C. L., J. Exper. Med., 108: 139, 1958.
3. MARKHAM, R., Biochem. J., 36: 790, 1942.
4. KABAT, E. A. AND MAYER, M. M., *Experimental Immunochemistry*, Charles C Thomas, Springfield, Ill., 1948.
5. FISCHEL, E. E., PAULI, R. H. AND LISH, J., J. Clin. Invest., 28: 1172, 1949.
6. OVARY, Z., Internat. Arch. Allergy, 3: 162, 1952.
7. ISHIZAKA, K. AND CAMPBELL, D. H., Proc. Soc. Exper. Biol. & Med., 97: 635, 1958.
8. COCHRANE, C. G. AND WEIGLE, W. O., J. Exper. Med., 108: 591, 1958.
9. ISHIZAKA, K., ISHIZAKA, T. AND CAMPBELL, D. H., J. Exper. Med., 109: 127, 1959.
10. VAUGHAN, J. H., J. Immunol., 77: 181, 1956.
11. EDELMAN, G. M., KUNKEL, H. G. AND FRANKLIN, E. C., J. Exper. Med., 108: 105, 1958.
12. BECKER, E. L., J. Cell. and Comp. Physiol., 50: 303, 1957.
13. BECKER, E. L., J. Allergy, 29: 191, 1958.
14. HUMPHREY, J. H. AND JAQUES, R., J. Physiol., 128: 9, 1955.
15. LEPOW, I. H., WURZ, L., RATNOFF, O. D. AND PILLEMER, L., J. Immunol., 73: 146, 1954.
16. LEVINE, L., Biochim. et biophys. acta, 18: 283, 1955.

17. Becker, E. L., Nature, **176:** 1073, 1955.
18. Lepow, I. H., Ratnoff, O. D., Rosen, F. S. and Pillemer, L., Proc. Soc. Exper. Biol. & Med., **92:** 32, 1956.
19. Geiger, W. B., J. Immunol., **69:** 597, 1952.
20. Ungar, G., Damgaard, E. and Hummel, F. P., J. Exper. Med., **98:** 291, 1953.
21. Plescia, O. J., Amirajan, K. and Cavallo, G., Fed. Proc., **16:** 429, 1957.
22. Bier, O. G., Siqueira, M. and Osler, A. G., Internat. Arch. Allergy, **7:** 1, 1955.
23. Osler, A. G., Hawrishiak, M. M., Ovary, Z., Siqueira, M. and Bier, O. G., J. Exper. Med., **106:** 811, 1957.

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THERMAL PROPERTIES OF HUMAN IgG

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Abstract—Dynamic light scattering experiments have been performed to study the aggregation kinetics of human immunoglobulin G (IgG). Aggregation and irreversible cluster growth results when IgG solutions (2–15 mg/ml) are heated above 50°C. The measured scattering intensity I and effective hydrodynamic radius $\langle R\rangle$ can be described consistently by a Smoluchowski aggregation process. The number of clusters $n_i(t)$ containing i monomers at time t are computed. The radius of an i cluster is assumed to be $R_i = R_0\, i^{\beta}$, where β is the cluster exponent. This kinetic process results in the following characteristic power law behavior: $\langle R\rangle/R_0 = (1 + \Gamma_R(T, C, \hat{c}), t)^{e_R}$ and $\langle I\rangle/I_0 = (1 + \Gamma_I(T, C, \hat{c})t)^{e_I}$. Here $R_0 = 5.51$ nm, is the monomer hydrodynamic radius, and I_0 the scattered intensity from the monomer solution at temperature T and conen C. A fraction, $\hat{c} \approx 0.48$ of the IgG monomers are heat stable up to 63°C and do not participate in the aggregation process. The power-law behavior of $\langle R\rangle/R_0$ and $\langle I\rangle/I_0$ indicates scaling, and indeed a very satisfactory data collapse results for our data. The best non-linear fit of the power-law forms gives $e_R = 0.48 \pm 0.05$, $e_I = 1.00 \pm 0.01$ and $\beta = 0.39 \pm 0.04$. We also find that the heat aggregation of IgG is an activated process. Fits of the experimental data gives Gibbs free energy for the activated complex $\Delta G^* = 13.8 \pm 0.1$ kcal/mole at 56°C. The temp dependence of the growth rates exhibits an Arrhenius behavior with an enthalpy of activation $\Delta H^* = 120 \pm 5$ kcal/mole.

INTRODUCTION

In diagnostic immunology, heating of serum at 56°C for 30 min has become a well established method for inactivating complement and removal of heat labile anticomplementary activity (Kwapinsky, 1972; Soltis *et al.*, 1979). This procedure usually does not influence the antibody activity or the other main biological properties of the immunoglobulins. On the other hand, heating of IgG in isolated form at 56°C leads to aggregation of the molecules and heating to 63°C for 15 min is a widely used method to produce soluble IgG aggregates. These aggregates possess many biological properties similar to antigen–antibody complexes: they fix complement, bind to macrophages, and can induce an Arthus reaction (Christian, 1960). They are therefore widely used as reactants for rheumatoid factor and as standards in various methods for immuno-complex assay (Frommhagen and Fudenberg, 1962; Ishizaka and Ishizaka, 1960; Henney and Stanworth, 4065; Augener and Grey, 1970; Nielsen and Svehag, 1976).

The temperature stability of IgG, the structure of the aggregates, and the mechanisms by which they form has been studied by several authors. (James *et al.*, 1964; Augener and Grey, 1970; Hansson, 1968; Oreskes and Mandel 1979, 1981, 1983; Zav'yalov *et al.*, 1975; McCarthy *et al.*, 1981*a*,*b*). The most common techniques in these studies have been analysis of hydrodynamic properties by gel filtration and ultracentrifugation. However, also light absorption, optical rotary dispersion, circular dichroism, thermal perturbation difference spectroscopy, solvent perturbation difference spectroscopy and difference adiabatic scanning microcalorimetry have given valuable information. When heated to about 60°C at low ionic strength, normal IgG forms soluble aggregates with sedimentation coefficients between 9.5 and 100 S, corresponding to mol. wts from 3×10^5 to 5×10^7 (Oreskes and Mandel, 1983). The tendency of IgG to aggregate when heated is not appreciably altered by the addition of 0.3 *M* NaCl, but in 2 *M* NaCl, no aggregation occurs (Frommhagen and Fudenberg, 1962). Also in the presence of serum albumin the aggregation processes are suppressed (Soltis *et al.*, 1979).

We have studied the kinetics of IgG heat aggregation by photon correlation spectroscopy. This technique measures the effective hydrodynamic radius $\langle R\rangle$ of the aggregates in solution. In conjunction with measurements of the scattered intensity $\langle I\rangle$, quite detailed information about the aggregation kinetics may be obtained. The method has been extensively described and reviewed in the literature (Berne and Pecora, 1976; Cummins, 1974; Chen *et al.*, 1981).

MATERIALS AND METHODS

Materials

Monomeric IgG was prepared from pooled human immunoglobulin G (Gammaglobulin Kabi 16%, AB Kabi, Stockholm, Sweden), by gel filtration on a Sephacryl S 300 superfine column. Gammaglobulin Kabi contains at least 97% IgG. Samples of Gam-

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maglobulin Kabi were eluted by a Tris–HCl buffer at pH = 7.6 (0.05 *M* Tris–HCl; 0.2 *M* NaCl; 2 m*M* EDTA; 0.02% NaN_3). From the monomeric fractions, samples were collected and passed through a 0.22 μm Millipore filter into thoroughly cleaned cylindrical glass cells. Four series of monomeric IgG with different concns (2, 4, 8 and 16 mg/ml) were prepared. The more conc. series were prepared by ultrafiltration in collodion bags. The protein concn was determined by OD measurements ($E^{1\%}_{1cm} = 14.0$ at 280 nm). Our results from dynamic light scattering demonstrated that the material was a monodisperse solution of molecules with an effective hydrodynamic radius R of 55.1 ± 0.3 Å. Eventually contamination with higher aggregates of IgG could easily be detected and such preparations were not used in the aggregation experiments.

A solution of monomeric ferritin molecules, for use as a reference standard, was prepared by gel filtration of horse spleen ferritin (Koch-Light, Colnbrooks, U.K.) on a Sepharose 6B column in the same buffer as above.

Dynamic light scattering

The light scattering experiments were done using a Malvern spectrometer (RR102). A He–Ne laser (Spectra Physics 124B) was used as a light source with the beam focused in the scattering cell. The scattered light was detected by the spectrometer photomultiplier and a digital correlator was used to measure the photocount correlation function from which the effective hydrodynamic radius $\langle R \rangle$ of the aggregating system can be obtained. The correlator was a 128 channel single clipped correlator built in this laboratory based on a design by Chen *et al.* (1975). A detailed description of the experimental design and the data analysis are given by Jessang *et al.* (1985).

We have tried to fit data with up to $n = 3$ components. We find, however, that with our low scattering intensities only fits with just one exponential give reliable results. We therefore take G, the photon correlation function decay rate, to give our best estimate of $\langle R \rangle$. We then evaluate the effective hydrodyanmic radius using the relation:

$$\langle R \rangle = kTQ^2/6\pi\eta G, \qquad (1)$$

$Q = (4\pi n/\lambda) \sin \theta/2$ is the magnitude of the scattering vector, where n is the refractive index of solution, λ is the wavelength of the laser, θ is the scattering angle and η is the solvent viscosity. Our standard scattering angle was $\theta = 50.0°$.

The samples, about 1 ml of solution, were filled into 10 mm diameter test tubes of Pyrex glass, which were used for scattering cells. The sample tubes were surrounded by a water bath for temp control and left undisturbed during the whole experiment. Temperature was controlled using an analog temp controller built in this laboratory. The temp stability was better than ±0.1°C in the full range of temps used in our experiments. In the aggregation experiments the desired temperature was first set and then the test tube with the sample was placed in the spectrometer. The temp of the sample increases rapidly and after about 1 min it reaches the bath temp. However, some convection in the scattering vol occasionally persists for several minutes. The data were corrected for temperature and concentration changes in viscosity and refractive index as described by Jessang *et al.* (1985).

In order to check the performance and calibration of our system we have measured $\langle R \rangle$ for spherical scatters as a function of scattering angle and temp. By scattering from a dilute solution of polystyrene latex spheres ($R = 54.5$ nm) we find that the measured $\langle R \rangle = (55.0 \pm 0.5)$ nm independent of scattering angle in the range $\theta = 30$–$150°$.

In order to have a calibration sample with spherical scatters of dimension close to those of IgG monomers we studied the scattering from monomeric ferritin. It was found that ferritin, an iron storage protein, is an unusually stable spherical protein. A 1 mg/ml solution in our standard Tris–HCl buffer is stable in the laboratory even at room temp for years. At room temp we find that $\langle R \rangle = (6.9 \pm 0.1)$ nm for all scattering angles between 30 and 150°. We conclude that unless we find this value for our ferritin standard, the spectrometer is out of alignment or unwanted reflections enter the detector.

RESULTS

Gel filtration

The heat-aggregation process was analyzed by analytical gel filtration on a Bio-Gel A-5m column. Heating at 59°C for 30 min results mainly in the formation of smaller aggregates as shown in Fig. 1(a). The amount of these oligomers decreased with increasing time, while the amount of higher polymers increased. After 6 hr heating at 62°C dimers were no longer detected by gel filtration while the amount of

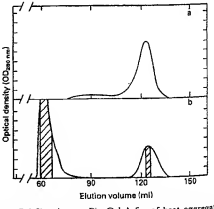

Fig. 1. Gel filtration on Bio-Gel A-5m of heat aggregated human IgG. (a) After 30 min at 59°C and (b) after 6 hr at 62°C.

polymers was high. A substantial amount of unaggregated monomers remains, Fig. 1(b). This fraction of monomers that do not participate in the aggregation process we designate "the heat stable fraction" (H-fraction). We estimate that approx. 50% of the monomers belong to the H-fraction.

Dynamic light scattering

Irreversible aggregation and thermal expansion. The polymer and monomer samples separated by gel filtration were studied separately by dynamic light scattering. The H-fraction had an effective hydrodynamic radius of $R = 5.83$ nm, slightly larger than that of the starting material at the same concn ($R = 5.68$ nm). The polymer peak in Fig. 1(b) contained particles with an average $\langle R \rangle$ in the range 160–210 nm. After storage for 10 days at room temp these aggregates were rechromatographed. Only traces of monomers were detected, indicating that the aggregation was mainly irreversible.

The effect of heating a sample of IgG at 8 mg/ml to 53°C can be seen in Fig. 2. The effective hydrodynamic radius $\langle R \rangle$ and the scattering intensity $\langle I \rangle$ both increased with time. After 20 hr at 53°C, the heater was turned off and the system cooled to 23°C in a few hours. As the system cooled the scattering intensity remained unchanged, whereas the radius $\langle R \rangle$ decreased from approx. 19 to 16 nm, and then remained constant. After about 75 hr from the initial heating the system was reheated to 39°C, the radius $\langle R \rangle$ increased to a new constant value, but the scattering intensity remained unchanged. Finally the sample was heated to 53°C and both $\langle R \rangle$ and $\langle I \rangle$ started to increase with time again as the aggregation process proceeded from where it was stopped by the first cooling step.

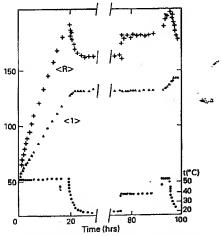

Fig. 2. The effective hydrodynamic radius $\langle R \rangle$ of IgG aggregates in Å +, and the scattering intensity $\langle I \rangle$ in arbitrary units ▲, as a function of time. The temperature record t is given by ● in °C.

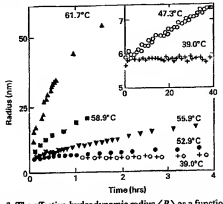

Fig. 3. The effective hydrodynamic radius $\langle R \rangle$ as a function of time for a 15.4 mg/ml sample of monomeric IgG at various temperatures.

The time dependence of $\langle R \rangle$. As the monomer solution of IgG was heated, aggregation started and the effective hydrodynamic radius $\langle R \rangle$ increased with time as shown in Fig. 3(a), the growth rate of $\langle R \rangle$ exhibited a very strong temp dependence. At 39°C, no aggregation was observed even after 80 hr. At 47°C the aggregation process had started, and at 62°C, very large clusters formed within an hour. The shape of the $\langle R \rangle$ (t) curves are very similar to those predicted for Smoluchowski aggregation (Smoluchowski, 1917). We therefore tried to fit the data at various concns and temps to the form

$$\langle R \rangle / R_0 = (1 + \Gamma_R C)^{\alpha_R}. \qquad (2)$$

Here we take R_0 to be the monomer radius as measured before the sample is heated. It was found that $\alpha_R = 0.50 \pm 0.06$, fits most of our data very well. In order to obtain the best estimates of the growth rate constant Γ_R as a function of temp and concn, α was taken to be 0.5, and fitted the observed $\langle R \rangle$ (t)

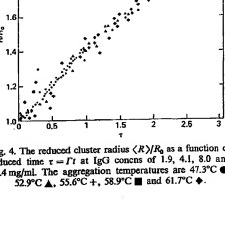

Fig. 4. The reduced cluster radius $\langle R \rangle / R_0$ as a function of reduced time $\tau = \Gamma t$ at IgG concns of 1.9, 4.1, 8.0 and 15.4 mg/ml. The aggregation temperatures are 47.3°C ●, 52.9°C ▲, 55.6°C +, 58.9°C ■ and 61.7°C ◆.

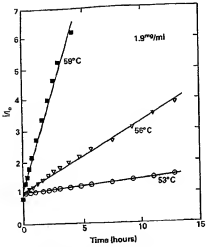

Fig. 5. The reduced scattering intensity I/I_0 as a function of time for a 1.9 mg/ml sample of monomeric IgG at various temperatures.

curves with Γ_R as the only free parameter. With the radius growth rate constants $\Gamma_R(C, T)$ determined by our fits to the data, we defined a reduced dimensionless time scale $\tau = \Gamma_R(C, T)t$ for each of the aggregation experiments. With this new time scale we obtained plots of $\langle R \rangle / R_0$ as a function of τ as shown in Fig. 4.

This way of plotting the results gives a very satisfactory data collapse, and within some experimental scatter all our results at concns of 1.9, 4.1, 8.0 and 15.4 mg/ml and temps of approximately 47, 53, 56, 59 and 62°C fall on a universal curve. We therefore conclude that the aggregation process proceeds with the power-law behavior of equation (2) characteristic of Smoluchowski aggregation kinetics. In order to get a more precise value for the radius growth exponent all the data in Fig. 4 was fitted

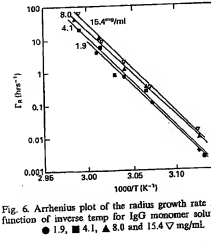

Fig. 6. Arrhenius plot of the radius growth rate Γ_R as a function of inverse temp for IgG monomer solutions at ● 1.9, ■ 4.1, ▲ 8.0 and 15.4 ▽ mg/ml.

with the expression (2) again and obtained $\alpha = 0.48 \pm 0.05$. This value of the radius exponent, unfortunately, does not determine the cluster exponent defined by $R/R_0 = i^\beta$, since approx. 50% of the monomers do not participate in the aggregation process. The non-participating fraction $\xi \approx 0.5$ has been determined by gel filtration and ultracentrifugation of samples that were held at 63°C for 6 hr. As shown by Jønsang *et al.* (1985), the cluster exponent β is estimated by

$$\beta = (\alpha - 0.015)/1.2 = 0.39.$$

The time dependence of $\langle I \rangle$. The scattering intensity grows linearly with time as seen in Fig. 5 for 1.9 mg/ml samples. The rate of intensity increase is strongly temp dependent, and we determined the intensity growth rate Γ as a function of temp T, and monomer concn C by fitting the expression

$$\langle I \rangle / I_0 = 1 + \Gamma_I t \tag{3}$$

to the data. Here I_0 is the intensity of the monomer solution before the heating process. In the same way as for $\langle R \rangle$ a complete data collapse is obtained by introducing a scaled time $\gamma = \Gamma_I t$ to the data. Since the intensities grow linearly with time all the relevant information is contained in the rate constants $\Gamma_I(C, T)$ as discussed in the next section.

Temp and concn dependence of the rate constants

The radius growth rate constant Γ_R increases exponentially with temp in a way characteristic of activated processes. In the Arrhenius plot of the growth rate constant (Fig. 6), we have also shown the fits of the form

$$\Gamma_R(T) = \Gamma_R^0 \exp(-\Delta H^*/R(1/T_0 - 1/T)). \tag{4}$$

Here ΔH^* is the activation enthalpy of the aggregation process, and Γ_R^0 is the radius growth rate constant at our standard reference temp $T_0 = 329.15$K, i.e. 56°C.

The intensity growth rate constant, Γ_I, has also been analyzed by Arrhenius plots with Γ_I replacing Γ_R in equation (4), and with the same observed linearity (results not shown). In Table 1, the results of this analysis are summarised. The parameters Γ_R, Γ_I, ΔH_R^* and ΔH_R^* and the standard deviation in these parameters have been estimated using a nonlinear least-squares minimization procedure fitting function 4 to the observed results.

The resulting values for ΔH^* (see Table 1), are independent of concn within our experimental accuracy. The average values are given in Table 2, and we find that by combining the results from measurements of $\langle R \rangle$ and $\langle I \rangle$ that our best estimate is $\Delta H^* = 120 \pm 5$ kcal/mole.

It is clear from Table 1 and Fig. 7 that the growth rates Γ_I and Γ_R at the standard temp increase with increasing concn. For Smoluchowski kinetics the collision rate constant $\gamma = 8\pi DRC_0$ is proportional to the monomer concn. We therefore fit the results in

Table 1. Kinetic parameters for IgG heat aggregation

C (mg/ml)	Γ_R^0 (hr^{-1})	$\Delta H_R^\ddagger$ (kcal/mole)	Γ_I^0 (hr^{-1})	$\Delta H_I^\ddagger$ (kcal/mole)
1.9	0.8 ± 0.05	-125 ± 2	0.26 ± 0.03	-113 ± 2
4.1	0.9 ± 0.15	-125 ± 3	0.4 ± 0.2	-114 ± 9
8.0	1.7 ± 0.20	-118 ± 8	0.72 ± 0.01	-115 ± 2
15.4	2.5 ± 0.10	-123 ± 2	1.2 ± 0.2	-123 ± 4

Table 2. Thermodynamic parameters for IgG heat aggregation

	ΔH^* (kcal/mole)	ΔG^* (kcal/mole)	σ (ml/mg hr)
$\langle R \rangle / R_0$	123 ± 3	13.5 ± 0.1	0.18 ± 0.01
$\langle I \rangle / I_0$	116 ± 5	14.5 ± 0.1	0.084 ± 0.005

Fig. 7 with the expression: $\Gamma = \sigma C_0$ with the results shown in Table 2. These σ coefficients may now be compared to the value expected from Smoluchowski kinetics. However, we must first correct for the presence of a non-aggregating monomer fraction. We have found that the non-aggregating fraction is ≈ 0.5 in our samples and with $\beta = 0.4$, as determined in the previous section, we conclude from Table 1 that the expected rate constants γ_R and γ_I equal 1.0 within a few per cent. Therefore, the observed growth rate constants Γ and Γ should satisfy the relation: $\Gamma_I / \Gamma_R = \Gamma_R / \tau = \gamma \tau$.

The "sticking coefficient", ϵ (that is the inverse number of collision on the average between two clusters before they irreversibly stick) is given by

$$\epsilon = (3\sigma\gamma M)/4RT, \tag{5}$$

where M is the mol. wt. With this expression we find $\epsilon = 1.13 \times 10^{-5}$ and 0.51×10^{-5} from the results for Γ_I and Γ_R.

DISCUSSION

Heating of monomeric IgG to above 50°C causes rapid aggregation and irreversible cluster growth.

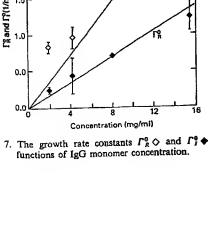

Fig. 7. The growth rate constants Γ_R^0 ◇ and Γ_I^0 ◆ as functions of IgG monomer concentration.

Figure 2 shows that these clusters are stable if cooled to room temp and continue to grow when reheated. The observed behavior of $\langle R \rangle$ and $\langle I \rangle$ is exactly what is expected for irreversible aggregation. As the system is cooled the aggregation process stops and the scattering intensity does not change since $\langle I \rangle$ essentially measures the number, i of molecules in the clusters $\langle I \rangle / I_0 = \Sigma_i n_i i^2$. We conclude that the size distribution $\{n_i\}$ must remain unchanged. If, for instance, the aggregates redissolve the scattering intensity would decrease. The change in $\langle R \rangle$ upon cooling and heating indicates that the clusters exhibit thermal expansion and is not a result of dissociation. We conclude that the thermal aggregation of IgG is an irreversible process, and that the aggregates are stable at room temp within the time period of our experiments. This result agrees well with the data of McCarthy *et al.* (1981*b*) and Oreskes and Mandel (1983) while Knutson *et al.* (1979) and Kauffman *et al.* (1979) report that human IgG preparations on heating gave rise to aggregates which were unstable. The differences in results are probably due to differences in procedure of preparation of AlgG as shown by McCarthy *et al.* (1981*a*). They compared different commercial IgG preparation and observed significant variation in stability of the aggregates. Aggregates formed by heating IgG from Kabi AB (Gammaglobulin Kabi) were also found stable in their study.

The observation that there was no aggregation even after 80 hr at 39°C is in disagreement with the report of James *et al.* (1964) who described the formation of up to 20% dimers after 20 hr at 37°C. Such dimerization, if present, would easily be detected by our method. Our data agree, however, with the observations of Mackay *et al.* (1973) that there was no change in the ultracentrifugation patterns of IgG, even after incubation at 37°C for 28 days. Also these differences may be due to differences in preparation methods.

The aggregation process, as seen by the scattering intensity and the effective hydrodynamic radius, can be described consistently as a Smoluchowski aggregation process (Smoluchowski, 1917; Jønsang *et al.*, 1985). This kinetic behavior results in a characteristic power law increase of the radius $\langle R \rangle$ and of the intensity $\langle I \rangle$ with time [equation (2) and (3)]. Because of this power-law behavior, the results may be scaled to produce the data collapse shown in Fig. 4. The radius growth exponent $\alpha = 0.48 \pm 0.05$ determines the cluster exponent β in the relation $R = R_0 i^\beta$ to be $\beta = 0.39 \pm 0.04$, when we take into account our

result that about 50% of the monomers are heat stable and do not participate in the observed aggregation process. The observation of a heat stable fraction is consistent with the experiments by Knutson *et al.* (1979), who found that at 63°C the maximum yield of aggregates was 50%.

The irreversible aggregation of clusters is an activated process. The temp dependence of the radius growth rate Γ_R and of the intensity growth rate Γ_I exhibits an Arrhenius behavior with an Arrhenius enthalpy of activation $\Delta H^* \approx 120$ kcal/mole. This value for ΔH^*, is in agreement with the typical values for protein denaturation (approximately 100 kcal/mole), (Haurowitz, 1963) and with the data for IgG aggregation reported by Oreskes and Mandel (1981) $\Delta H = 130$ kcal/mole.

We have found that the Smoluchowski sticking coefficient ϵ is very small, approx. equal to 10^{-3}. Since the aggregation process is an activated process only a fraction of the collisions can be successful. We expect that only certain relative configurations and relative orientations of the colliding clusters may lead to a permanent bond. In addition even if the relative orientation is acceptable the cluster may have to overcome a potential barrier, possibly including the rupture of weak bonds, before the stable configuration is reached. In thermodynamic terms these factors are expressed by Gibbs free energy (ΔG^*) of the system as a whole when two clusters are put together into a state where the resulting complex may either bind irreversibly or split apart with equal probability. The sticking coefficient ϵ is related to ΔG^* by $\epsilon = \exp(-\Delta G^*/RT_0)$. With this expression we may use the value for ϵ determined from equation (5) to estimate ΔG^* with the results shown in Table 2. It should be stressed that ΔG^* contains entropy terms that stem from the orientation of the clusters as well as from the configurations of the complex.

The process leading to heat aggregation of IgG is not fully understood. Increasing temperature would be expected to increase hydrophobic interactions (Kauzmann, 1959). Presumably heating results in the rupture of several intra-molecular bonds leading to a partly denatured molecule. Observations of the changes in optical rotation indicate that denaturation starts at about 47°C for IgG (Henney and Stanworth, 1965). Zav'yalov *et al.* (1975) found from thermal perturbation difference spectra, reversible structural changes in a myeloma IgG preparation between 25 and 33°C and that denaturation occurred in a narrow temp interval at 64°C. The denatured molecules contain probably unsatisfied potential bonding points. Therefore, if the molecules come in contact, some or all of the ruptured bonds may reform inter-molecularly and aggregates are formed. We note, however, that there is no indication of a denaturation process in our results. If there is a denaturation step below 50°C, it must be a conformational change in the molecules that leaves the effective hydrodynamic radius unchanged. Of course, at higher temps a significant denaturation step would be masked by the rapid aggregation process.

In the aggregation of serum albumin the importance of disulfide bond formation has been demonstrated (Frensdorff *et al.* 1953). Our experiments, however, indicate that this is not an important factor in the heat aggregation of IgG, since addition of iodoacetamide had no effect on the aggregation rate. Iodoacetamide is known to react with free–SH groups and to prevent disulfide bond formation. This observation is consistent with the results presented by Augener and Grey (1970).

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REFERENCES

Augener W. and Grey H. (1970) Studies of the mechanism of heat aggregation of human IgG. *J. Immun.* 105, 1024–1030.

Berne B. J. and Pecora R. (1976) *Dynamic Light Scattering*. John Wiley, New York.

Chen S. H., Veldkamp W. B. and Lai C. C. (1975) Simple digital clipped Correlator for photon correlation spectroscopy. *Rev. Sci. Instrum.* 46, 1356–1364.

Chen S. H., Chu B. and Nossal R. (1981) In *Scattering Techniques Applied to Supramolecular and Nonequilibrium Systems*. Plenum Press, New York.

Christian C. L. (1960) Studies on aggregated gamma-globulin I & II. *J. Immun.* 84, 112–121.

Cummins H. Z. (1974) *Photon Correlation and Light Beating Spectroscopy*. Plenum Press, New York.

Frensdorff H. K., Watson M. T. and Kauzmann W. (1953). The kinetics of protein denaturation V. The viscosity of urea solutions of serum albumin. *J. Am. chem. Soc.* 75, 5167–5172.

Frommhagen L. E. and Fudenberg H. (1962) The role of aggregated γ-globulins in the anticomplementary activity of human and animal sera. *J. Immun.* 89, 336–343.

Hansson U. B. (1968) Ultracentrifugal studies of the aggregation of human Immunoglobulin G by freezing and by heating. *Acta chem. scand.* 22, 953–960.

Haurowitz F. (1963) In *The Chemistry and Function of Proteins* (2nd edn), pp. 161–174. Academic Press, New York.

Henney C. S. and Stanworth D. R. (1965) The reactivity of Rheumatoid factor with human gamma G globulin. *Immunology* 9, 139–150.

Ishizaka K. and Ishizaka T. (1960) Biological activity of aggregated gamma-globulin. II. A study of various methods for aggregation and species differences. *J. Immun.* 85, 163–171.

James K., Henney C. S. and Stanworth D. R. (1964) Structural changes occurring in 7S γ-globulins. *Nature, Lond.* 202, 563–566.

Jansaag T., Feder J. and Rosenqvist E. (1985) Heat aggregation kinetics of human IgG. *J. chem. Phys.* 82, 574–580.

Kaufmann R. H., van Es L. A. and Daha M. R. (1979) Aggregated human immunoglobulin G stabilized by albumin: a standard for immune complex detection. *J. Immun. Meth.* 31, 11–22.

Kauzmann W. (1959) Some factors in the interpretation of protein denaturation. *Adv. protein Chem.* **14**, 1–63.

Knutson D. W., Kijlstra A., Lentz H. and van Es L. A. (1979) Isolation of stable aggregates of IgG by zonal ultracentrifugation in sucrose gradients containing albumin. *Immun. Commun.* **8**, 337–345.

Kwapinsky J. B. G. (1972) The complement fixation test. In *Methodology of Immunochemical Research*, pp. 482–524. Wiley–Interscience, New York.

Mackay M., Vallet L. and Combridge B. S. (1973) The characterisation and stability during storage of human immunoglobulin prepared for clinical use. *Vox Sang.* **25**, 126–140.

McCarthy D., Goddard D. H., Embling P. H. and Holborrow E. J. (1981*a*) A simple procedure for assessing the stability of heat aggregated IgG preparations. *J. Immun. Meth.* **41**, 75–79.

McCarthy D., Goddard D. H., Pell B. K. and Holborow E. J. (1981*b*) Intrinsically stable IgG aggregates. *J. Immun. Meth.* **41**, 63–74.

Nielsen H. and Svehag S. E. (1976) Detection and differentiation of immune complexes and IgG aggregates by a complement consumption assay. *Acta path. microbiol. scand.* **C84**, 261–269.

Oreskes I. and Mandel D. (1979) Immunological properties of aggregated human IgG: Column fractionation of thermal aggregates. In *Proteides of the Biological Fluids* (Edited by Peeters H.), pp. 107–110. Pergamon Press, Oxford.

Oreskes I. and Mandel D. (1981) Kinetic studies of IgG aggregation. *Rev. du Rhumatisme*, Paris. Num. spec. Juin 1981. Abstract 0633.

Oreskes I. and Mandel D. (1983) Size fractionation of thermal aggregates of immunoglobulin G. *Analyt. Biochem.* **134**, 199–204.

Smoluchowski M. V. (1917) Versuch einer matematischen theorie der koagulationkinetik koloider lösungen. *Z. phys. Chem.* **92**, 129–168.

Soltis R. D., Hasz D., Morris M. J. and Wilson I. D. (1979) The effect of heat inactivation of serum on aggregation of immunoglobulins. *Immunology* **36**, 37–45.

Zav'yalov V. P., Troitsky G. V., Demchenko A. P. and Generalov I. V. (1975) Temperature and pH dependent changes of immunoglobulin G structure. *Biochim. biophys. Acta* **386**, 155–167.

A Sudden Decline in Active Membrane-Bound TGF-β Impairs Both T Regulatory Cell Function and Protection against Autoimmune Diabetes[1]

Randal K. Gregg,[2] Renu Jain, Scott J. Schoenleber, Rohit Divekar, J. Jeremiah Bell, Hyun-Hee Lee, Ping Yu, and Habib Zaghouani[3]

Autoimmunity presumably manifests as a consequence of a shortfall in the maintenance of peripheral tolerance by $CD4^+CD25^+$ T regulatory cells (Tregs). However, the mechanism underlying the functional impairment of Tregs remains largely undefined. In this study a glutamic acid decarboxylase (GAD) diabetogenic epitope was expressed on an Ig to enhance tolerogenic function, and the resulting Ig-GAD expanded Tregs in both young and older insulitis-positive, nonobese diabetic (NOD) mice, but delayed autoimmune diabetes only in the former. Interestingly, Tregs induced at 4 wk of age had significant active membrane-bound TGF-β (mTGF-β) and sustained protection against diabetes, whereas Tregs expanded during insulitis had minimal mTGF-β and could not protect against diabetes. The Tregs probably operate suppressive function through mTGF-β, because Ab blockade of mTGF-β nullifies protection against diabetes. Surprisingly, young Tregs that modulated pathogenic T cells maintained stable frequency over time in the protected animals, but decreased their mTGF-β at the age of 8 wk. More strikingly, these 8-wk-old mTGF-β-negative Tregs, which were previously protective, became unable to confer resistance against diabetes. Thus, a developmental decline in active mTGF-β nullifies Treg function, leading to a break in tolerance and the onset of diabetes. ***The Journal of Immunology*****, 2004, 173: 7308–7316.**

Recently, it has become clear that $CD4^+CD25^+$ T regulatory cells (Tregs)[4] play a major role in the maintenance of peripheral tolerance (1, 2). Currently, a tremendous effort is being deployed to understand how these cells develop and exercise suppressive function against hazardous self-reactive T lymphocytes (3–5). In the nonobese diabetic (NOD) mouse, activation of pathogenic T cells, the presumed triggers of spontaneous diabetes in these animals, is viewed as a breakdown of Treg-mediated peripheral tolerance (6, 7). Initially, a decrease in the frequency of Tregs was suggested for the susceptibility of the NOD mouse to diabetes (7). Recently, however, it has been reported that the number of Tregs is steady over the course of disease (8), but a loss of function was observed and correlated with the onset of diabetes (9). The mechanism underlying such acquired ineffectiveness remains largely undefined. Membrane-bound TGF-β (mTGF-β) on Tregs has recently been shown to mediate cell contact inhibition of pathogenic T cells (10) and play a critical role in Treg suppressive function (11–13). In fact, anti-islet $CD8^+$ T cells expressing a dominant negative TGF-β receptor transgene could not be targeted by Tregs in vivo (14). In this study an approach for peptide delivery on Ig was developed, and a treatment regimen was defined that expanded mTGF-β-positive Tregs and protected animals against diabetes. Moreover, we found that an abrupt decline in mTGF-β expression on Tregs accompanied by a loss of suppressive functions transpire during the transition to destructive insulitis and progression to diabetes. Indeed, when the glutamic acid decarboxylase (GAD65) 524–543 peptide (designated GAD1) (15, 16) was genetically engineered into an Ig molecule, the resulting Ig-GAD1 expanded Tregs expressing active mTGF-β and protected young mice against diabetes. However, Ig-GAD1 given to 8-wk-old mice with progressive insulitis induced Tregs lacking mTGF-β and did not protect against diabetes. Interestingly, 6-wk-old Tregs, whether from Ig-GAD1 treated or naive NOD mice, expressed mTGF-β and delayed diabetes when cotransferred with diabetogenic splenocytes into NOD.*scid* mice. However, 8- or 26-wk-old Tregs, whether from naive or Ig-GAD1-treated nondiabetic animals, had minimal mTGF-β and could not protect NOD.*scid* mice against passive diabetes. Furthermore, blockade of mTGF-β with Abs before transfer into NOD.*scid* mice nullifies the protective function of the otherwise suppressive 6-wk-old Tregs. Together, these results indicate that a decline in cell surface expression of active TGF-β during transition to insulitis is responsible for the loss of suppressive function of Tregs and the resulting onset of diabetes.

Materials and Methods

Mice

NOD ($H\text{-}2^{g7}$) and NOD.*scid* mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-4-deficient ($IL\text{-}4^{-/-}$) and IL-10-deficient ($IL\text{-}10^{-/-}$) NOD mice were previously described (17, 18). All mice were

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[4] Abbreviations used in this paper: Treg, T regulatory cell; agg, aggregated; C_T, threshold cycle; Foxp3, Forkhead/winged helix transcription factor gene; GAD65, glutamic acid decarboxylase-65; HEL, hen egg lysozyme; IAA, insulin autoantibody; INSβ, insulin β-chain; mTGF-β, membrane-bound TGF-β; nil, untreated; NOD, nonobese diabetic; sol, soluble; T1D, type I diabetes.

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maintained in the animal facility for the duration of experiments, and the experimental procedures performed on these animals were conducted according to the guidelines of the institutional animal care and use committee.

Assessment of diabetes

Mice were bled from the tail vein weekly, and the blood samples were used to assess both glucose content and anti-insulin Abs. For measurement of glucose, a drop of blood was directly placed on a test strip, and the glucose content was read using a FreeStyle blood glucose-monitoring system (TheraSense, Alameda, CA). For detection of anti-insulin Abs, the blood was allowed to coagulate for 1 h at room temperature, and the serum was separated and used for ELISA. A mouse was considered diabetic when the blood glucose was >300 mg/dl for 2 consecutive weeks.

Antigens

Peptides. All peptides used in this study were purchased from Metabion (Munich, Germany) and were purified by HPLC to >90% purity. Insulin β-chain (INSβ) peptide (SHLVEALYLVCGERG) encompasses a diabetogenic epitope corresponding to aa residues 9–23 of INSβ (19). GAD1 peptide (SRLSKVAPVIKARMMEYGTT) corresponds to aa residues 524–543 of GAD65 (20, 21). GAD2 peptide (TYEIAPVFVLLEYVT) corresponds to aa residues 206–220 of GAD65 (22). Hen egg lysozyme (HEL) peptide (AMKRHGLDNYRGYSL) encompasses a nondiabetogenic epitope corresponding to aa residues 11–25 of HEL (23). INSβ, GAD1, GAD2, and HEL peptides are presented to T cells in association with I-A^{g7} MHC class II molecules.

Ig chimeras. Ig-GAD1 and Ig-HEL are chimeras expressing GAD1 and HEL peptides, respectively. Insertion of GAD1 and HEL nucleotide sequences into the CDR3 of the H chain variable region of 91A3 IgG2b, κ Ig, was conducted as previously described (24). Large-scale cultures of transfectoma cells were used in DMEM containing 10% iron-enriched calf serum (BioWhittaker, Walkersville, MD). Purification of Ig-GAD1 and Ig-HEL was conducted on separate columns of rat anti-mouse κ-chain mAb coupled to cyanogen bromide-activated 4B Sepharose (Amersham Biosciences, Piscataway, NJ). Aggregation of the Ig chimeras was conducted by precipitation with 50% saturated $(NH_4)_2SO_4$ as previously described (24).

Generation of T cell clones

A T cell clone specific for GAD1 peptide was generated by immunizing NOD mice with 50 μg of GAD1 peptide in 200 μl of PBS/CFA (v/v) s.c. in the footpads and at the base of each limb. After 10 days, the draining lymph nodes were removed, and T cells were stimulated in vitro for two rounds in the presence of irradiated (3000 rad), syngeneic splenocytes, 5% T-Stim supplement (Collaborative Biomedical Products, Bedford, MA), and GAD1 peptide (15 μg/ml). Cloning of a T cell line specific for GAD1 was accomplished by limiting dilution. The culture medium used to carry out these stimulations and other T cell activation assays in this study was DMEM supplemented with 10% FCS (HyClone, Logan, UT), 0.05 mM 2-ME, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μg/ml gentamicin sulfate.

Isolation of T cells

$CD4^+$ T lymphocytes were isolated from the spleen by positive selection on microbeads (Miltenyi Biotech, Auburn, CA). For $CD4^+CD25^+$ T cells, splenic cells were depleted of RBC, and $CD4^+$ lymphocytes were separated by negative selection using the Miltenyi CD4 T cell isolation kit. The $CD4^+CD25^+$ T cells were isolated by positive selection using anti-CD25-coupled Miltenyi microbeads. The CD25-negative fraction ($CD4^+CD25^-$) was used as a control for $CD4^+CD25^+$ T cells. All procedures were conducted according to Miltenyi's instructions.

Isolation of BSA-APCs

Partial purification of splenic APC was accomplished by floating fresh NOD spleen cells on a dense BSA gradient, and the cells were then washed in plain culture medium and used in T cell activation assays.

Flow cytometric analyses

For staining of CD4, CD25, and CD62L, purified splenic $CD4^+$ T cells (1.5×10^6) were incubated with anti-CD4-PE, anti-CD25-allophycocyanin (or isotype control rat IgG1-allophycocyanin), and anti-CD62L-FITC (or isotype control rat IgG2a-FITC) for 30 min at 4°C and washed with buffer. The cells were fixed with 2% formaldehyde for 20 min at room temperature and then analyzed. Events ($30–50 \times 10^6$) were collected on a FACSVantage flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using CellQuest software 3.3 (BD Biosciences). Staining for CTLA-4 was conducted as follows: purified islet and splenic $CD4^+$ T cells (2×10^6 cells) were incubated with anti-CTLA-4-PE (4F10) or isotype control hamster IgG1-PE for 2 h at 37°C, followed by anti-CD4-FITC and anti-CD25-allophycocyanin or isotype control rat IgG1-allophycocyanin for 30 min at 4°C. The cells were then washed, fixed with 2% formaldehyde, and analyzed as described above. Anti-CD4-FITC or -PE, anti-CD25-allophycocyanin, anti-CD62L-FITC, anti-CTLA-4-PE, rat IgG1-allophycocyanin, rat IgG2a-FITC, and hamster IgG1-PE were purchased from BD Pharmingen (San Diego, CA). Staining for surface expression of active TGF-β was conducted as previously described (10). Briefly, purified $CD4^+$ T cells (2×10^6 cells) were incubated with anti-CD4-FITC, anti-CD25-allophycocyanin (or isotype control rat IgG-allophycocyanin), and biotin-conjugated anti-TGF-β1 (BAF240) or with isotype control chicken IgY-biotin for 30 min at 4°C and washed with buffer. Subsequently, the cells were stained with PE-conjugated streptavidin for 30 min at 4°C. The cells were then washed, fixed with 2% formaldehyde, and analyzed as described above. Biotin-conjugated anti-TGF-β1 and chicken IgY were purchased from R&D Systems (Minneapolis, MN).

Proliferation assays

For presentation of Ig-GAD1 to a specific T cell clone, irradiated (3000 rad) NOD splenocytes (5×10^5 cells/50 μl/well) were incubated with 100 μl of Ag, and 1 h later, GAD1-specific T cells, TCC-GAD1-1F6 (5×10^4 cells/well/50 μl), were added. After 3-day incubation, 1 μCi of [^{3}H]thymidine was added per well, and the culture was continued for an additional 14.5 h. The cells were then harvested on a Mach III harvester (Tomtec, Hamden, CT), and incorporated [^{3}H]thymidine was counted on a Trilux 1450 Microbeta counter (Wallac, Gaithersburg, MD) using Microbeta 270.004 software (Wallac). For activation of splenic T cells after Ig chimera treatment, purified $CD4^+$ T cells (2.5×10^5 cells/well) were incubated with irradiated (3000 rad) BSA-APCs (5×10^5) and 20 μg/ml peptide for 72 h. After the incubation, 1 μCi of [^{3}H]thymidine was added per well, and the culture was continued for an additional 14.5 h. The cells were then harvested and counted as described above. For alloantigen-induced expansion, isolated $CD4^+CD25^-$ T cells (2×10^5 cells/well) were incubated for 5 days with T cell-depleted C57BL/6 splenic cells (1×10^5 cells/well) and increasing numbers of $CD4^+CD25^+$ T cells. The $CD4^+CD25^+$ T cells were incubated for 2 h with or without anti-TGF-β Ab (1D11) or mouse IgG isotype control and washed before addition to the alloantigen reaction mix. The culture was pulsed 8 h before harvesting with 1 μCi of [^{3}H]thymidine and then counted.

Cytokine production by Tregs

Splenic $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells (2.5×10^5 cells/well) were stimulated with 30 μg/ml peptide for 48 h in the presence of irradiated (3000 rad) BSA-APCs (5×10^5 cells/well). Subsequently, cytokine production was assessed by ELISA from 100 μl of culture supernatant.

Detection of cytokines in cell cultures

Detection of IL-10 was conducted by ELISA according to BD Pharmingen's standard protocol. The capture Ab was rat anti-mouse IL-10, JES5-2A5, and the biotinylated anti-cytokine Ab was rat anti-mouse IL-10, JES5-16E3. Both Abs were purchased from BD Pharmingen. Detection of TGF-β was performed according to the procedure outlined by R&D Systems. To activate latent TGF-β to the immunoreactive form, samples were acidified by the addition of HCl (20 mM) for 10 min at room temperature, then neutralized by NaOH/HEPES solution. The capture Ab was mouse anti-TGF-β1, -β2, and -β3 1D11 mAb, and the biotinylated anti-cytokine Ab was chicken anti-TGF-β1 (BAF240). Both Abs were purchased from R&D Systems. All assays were read on a SpectraMAX 190 counter (Molecular Devices, Sunnyvale, CA) and analyzed using SOFTmax PRO 3.1.1 software. Graded amounts of recombinant mouse IL-10 (BD Pharmingen) and TGF-β (R&D Systems) were included in all experiments for construction of standard curves. The cytokine concentration in culture supernatants was extrapolated from the linear portion of the standard curve.

Depletion of Tregs

For depletion of $CD4^+CD25^+$ T cells in vivo, mice were injected with 1 mg of anti-CD25 Ab (PC61)/mouse alone or in conjunction with aggregated (agg) Ig-GAD1 treatment. Rat IgG (1 mg/mouse) was used as a control.

Suppression of passive diabetes by Tregs

Splenic cells were harvested from untreated (nil) and Ig chimera-treated mice at the ages indicated. Subsequently, splenic $CD4^+CD25^+$ and

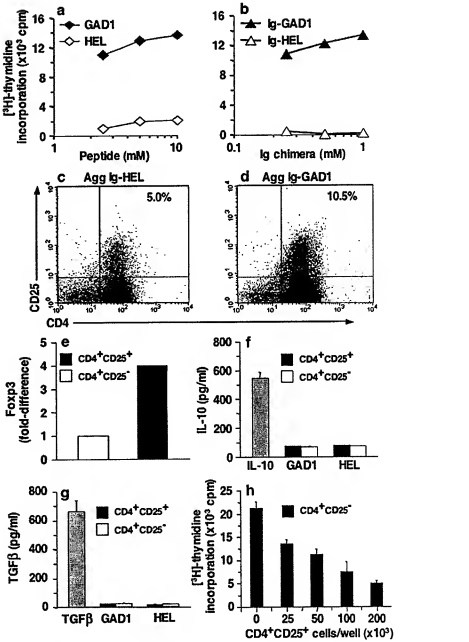

FIGURE 1. Agg Ig-GAD1 induces nonproliferative $CD4^+CD25^-$ T cells, expressing Foxp3, but not secreting IL-10 or TGF-β. Irradiated NOD APC splenocytes were incubated with free peptides (*a*) or Ig-chimeras (*b*); 1 h later, GAD1 peptide-specific T cells were added. T cell activation was assessed by [^{3}H]thymidine incorporation after a 72-h incubation. HEL peptide and Ig-HEL were included for negative control purposes. For expansion of Tregs, female NOD mice were given an i.p. injection of 300 μg of agg Ig-HEL (*c*) or Ig-GAD1 (*d–h*) at 4, 5, and 6 wk of age. Phenotypic and functional analyses were performed 7 days after the last injection. *c* and *d*, Splenic cells were analyzed for CD4 and CD25 expression by flow cytometry. *e*, Foxp3 expression was assessed by real-time PCR using the comparative C_T method. IL-10 (*f*) and TGF-β (*g*) secretion by $CD4^+CD25^+$ vs $CD4^+CD25^-$ T cells was determined by ELISA after a 48-h Ag stimulation. Recombinant IL-10 and TGF-β (▨) were used as controls. *h*, Proliferation of the $CD4^+CD25^-$ fraction (2×10^5 cells/well) was assessed by [^{3}H]thymidine incorporation after 5-day incubation in the presence of allogeneic C57BL/6 splenocytes (1×10^5 cells/well) alone or together with increasing numbers of $CD4^+CD25^-$ counterparts. Each bar represents the mean ± SD of triplicate wells.

$CD4^+CD25^-$ T cells were purified and resuspended in PBS. Additionally, spleens from recently diabetic NOD female mice (~2 wk diagnosed) were harvested, and the isolated diabetogenic splenocytes (used to induce diabetes in NOD.*scid*) were resuspended in PBS. Then, 5×10^5 cells $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were coinjected i.v. with 1×10^7 diabetogenic splenocytes into NOD.*scid* mice (4–8 wk of age). In some experiments the $CD4^+CD25^+$ T cells were incubated for 2 h with 100 μg/ml anti-TGF-β (1D11) or isotype control mouse IgG before cotransfer with diabetogenic splenocytes into NOD.*scid* mice.

Real-time PCR for Foxp3 expression

Total RNA was extracted from cells using TRIzol reagent. RT and DNA amplification were performed according to the one-step protocol using 300 ng of total RNA and a QuantiTect SYBR Green real-time PCR kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Primer pairs were as follows: Foxp3, 5′-GGCCTTCTCCAGGACAGA-3′ and 5′-GCT GATCATGGCTGGGTTGT-3′; and β-actin, 5′-AGAGGGAAATCGT GCGTGAC-3′ and 5′-CAATAGTGATGACCTGGCCGT-3′. Real-time PCR was performed on a Cepheid Smart Cycler (Sunnyvale, CA), and the results were analyzed by the comparative threshold cycle (C_T) method using Smart Cycler software. The comparative C_T method allows relative quantitation of gene expression to be performed where C_T represents the cycle where detection of an increased signal associated with exponential growth of PCR product begins. Furthermore, ΔC_T values represent the difference between a sample C_T and a normalizer C_T such as β-actin. For comparisons of gene expression, the $\Delta\Delta C_T$ values are used and represent the difference between the sample ΔC_T and a reference ΔC_T. Finally, quantitation using the formula $2^{-\Delta\Delta C_T}$ provides a comparative expression level for comparisons of differing conditions, such as treatments or ages. This comparative expression level, therefore, represents a fold difference from that of the reference level.

Insulin autoantibody assay

Detection of insulin autoantibodies (IAA) in the serum of NOD mice was conducted by ELISA as follows. Microtiter plates (no. 3369; Corning Glass, Corning, NY) were coated with 50 μl of sodium bicarbonate solution (pH 9.6) containing 10 μg/ml porcine insulin (Sigma-Aldrich, St. Louis, MO) for 16 h at 4°C. The plates were then washed three times with PBS-0.05% Tween 20, and free plastic sites were saturated by incubation with 2.5% casein (in 0.3 M NaCl, pH 7) for 2 h at room temperature.

Subsequently, serum samples (1/200 dilutions) were added, and the plates were incubated for 16 h at 4°C. Biotin-conjugated, rat anti-mouse κ mAb (100 μl at 1 μg/ml) was added, and the plates were incubated for 1 h at room temperature. Bound anti-mouse κ mAb was revealed by incubation with a casein solution containing 2.5 mg/ml avidin peroxidase for 30 min at room temperature, followed by addition of ABTS substrate. The samples were read at 405 nm on a Spectramax 190. A sample is considered IAA positive when the OD_{405} is >0.2. This cutoff line of 0.2 was chosen because serum samples from 10 SJL mice, which are not prone to diabetes development and presumably do not produce insulin-specific autoantibodies, never exceeded 0.05 OD_{405} (4-fold less than cutoff).

Statistical analysis

The χ^2 test was used for data analysis among experimental and control groups. Cytokine levels were compared using Student's *t* test for unpaired samples.

Results

Agg Ig-GAD1 expands Tregs protective against diabetes

T cells made against GAD1 peptide proliferated upon stimulation with GAD1, but not the negative control HEL peptide (Fig. 1*a*). Ig-GAD1, but not the negative control Ig-HEL, was presented to these specific cells and induces their proliferation (Fig. 1*b*). These data indicated that Ig-GAD1 is internalized by APCs, and the GAD1 peptide is released and presented to the T cells in a specific manner. It was recently shown that aggregation of Ig-myelin chimeras cross-linked FcγR on APCs and increased the myelin peptide's tolerogenic functions (24–26). Whether such regimens operate through expansion of Tregs is unknown. Administration of agg Ig-GAD1 into young NOD mice induced the expansion of cells with a regulatory phenotype. Indeed, the number of $CD25^+$ T cells among all $CD4^+$ T lymphocytes rose from 5.0% in the agg Ig-HEL-treated mice to 10.5% in the animals given agg Ig-GAD1 (Fig. 1, *c* and *d*). Nil or soluble (sol) Ig-GAD1-treated mice had 4–6% $CD4^+CD25^+$ T cells (data not shown). These $CD4^+CD25^+$ T cells had an increased mRNA expression of the Forkhead/winged helix transcription factor (Foxp3) gene relative to their $CD4^+CD25^-$ counterparts (Fig. 1*e*), concurring with a Treg phenotype (27, 28). These Tregs did not secrete IL-10 or TGF-β (Fig. 1, *f* and *g*), but displayed significant suppressive functions against their $CD4^+CD25^-$ counterparts (Fig. 1*h*). Without a doubt, the $CD4^+CD25^-$ T cells mounted significant MLR proliferation against T cell-depleted allogeneic C57BL/6 splenocytes, but a marked decrease in the proliferation was observed when $CD4^+CD25^+$ T cells were added to the culture (Fig. 1*h*). Thus, treatment with agg Ig-GAD1 resulted in expansion of T cells with both phenotypic and functional marks of Tregs. Additional in vivo analyses seem to associate these Tregs with a significant delay of diabetes. In fact, mouse recipients of agg Ig-GAD1 treatment reduced their spontaneous proliferative responses to diabetogenic peptides such as INSβ, GAD1, and GAD2 in comparison with animals recipient of the control molecule agg Ig-HEL (Fig. 2*a*). It should be noted that HEL peptide, although restricted to $I\text{-}A^{\beta7}$-like GAD1 peptide, is not a self-determinant, and NOD mice do not develop spontaneous responses against it. Thus, the lack of proliferation against HEL peptide in Ig-GAD1- and Ig-HEL-treated mice is due to the absence of a spontaneous response, rather than to down-regulatory functions by the chimeras. Furthermore, a significant level of protection against diabetes was observed in these animals. Indeed, only 40% of mice treated with agg Ig-GAD1 developed diabetes compared with 70% of control agg Ig-HEL treated and 80% of nil animals (Fig. 2*b*). It should be noted that some protection was seen with the control agg Ig-HEL in the early stage of disease, which is probably due to bystander suppression

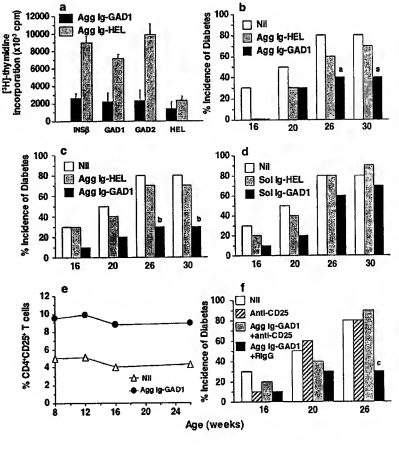

FIGURE 2. Agg Ig-GAD1 induces Tregs and delays diabetes in young NOD mice. Female NOD mice (10/group) were given i.p. 300 μg of either agg Ig-GAD1 or Ig-HEL at 4, 5, and 6 wk of age without (*a*, *b*, and *e*) or with (*f*) 1 mg of anti-CD25 Ab or rat IgG isotype control. *a*, Mice were killed at wk 12, and their splenic proliferation against the indicated peptides was assessed by [^{3}H]thymidine incorporation as described in *Materials and Methods*. The bars represents the mean ± SD of triplicate wells. *b–d* and *f*, Mice were monitored for blood glucose up to wk 26 or 30 of age. *c* and *d*, Mice were given weekly i.p. injection of 300 μg of sol or agg Ig-GAD1 (■) or Ig-HEL (▨) beginning at wk 4 until wk 12. Biweekly injections were then applied until wk 26 of age. *c* and *d*, Blood glucose was monitored weekly up to wk 30. *e*, A group of nondiabetic mice was killed at the indicated week and used for evaluation of $CD4^+CD25^+$ T cell percentages by flow cytometry. A group of mice that did not receive any injection (Nil) was included to serve as a control in all experiments. *a*, $p < 0.05$; *b*, $p < 0.01$; *c*, $p < 0.01$ (compared with nil group).

by IL-10 produced by the APCs upon cross-linking of FcγRs (24). The sol form of Ig-GAD1, which did not expand $CD4^+CD25^+$ Tregs, supported a delay in disease onset through 20 wk of age (10% incidence of diabetes vs 50% for both nil and agg Ig-HEL). However, the incidence of disease rose to 80%, which is similar to that in the control sol Ig-HEL-treated group (data not shown). A prolonged treatment regimen, consisting of a weekly injection of 300 μg of agg chimeras from wk 4–12 and biweekly injections thereafter until wk 30 of age, produced only a slight enhancement of disease prevention; 30% of the mice became diabetic by wk 30 (Fig. 2*c*) vs 40% in the short treatment group (Fig. 2*b*). The nil as well as agg Ig-HEL groups displayed similar incidences of disease as the short treatment regimen (Fig. 2, *b* and *c*). A prolonged regimen with sol Ig-GAD1 remains less effective, because only transient protection was observed at wk 20, and most of the mice became diabetic by wk 26 of age (Fig. 2*d*). Hence, the results indicate that a short treatment at the preinsulitis stage is sufficient to induce optimal protection by agg Ig-GAD1. The delay of disease onset is most likely controlled by Tregs. This statement stems from the observation that the percentage of $CD4^+CD25^+$ T cells was maintained at expanded (10% of total $CD4^+$ cells) levels through 26 wk of age relative to the 5% obtained with the untreated mice (Fig. 2*e*). Moreover, depletion of these Tregs at the preinsulitis stage nullified the suppressive effects of agg Ig-GAD1. Indeed, 90% of the mice given anti-CD25 Ab during treatment with agg Ig-GAD1 became diabetic by wk 26 of age, whereas only 30% of the animals displayed hyperglycemia when rat IgG replaced anti-CD25 Ab (Fig. 2*f*). Interestingly, anti-CD25 Ab alone did not affect the pattern of disease, indicating that interference with activated pathogenic T cells was minimal. Overall, these results indicate that agg Ig-GAD1 expands cells with a phenotypic pattern characteristic of T regulatory cells and operates protection against diabetes through the suppressive function of these Tregs.

In a number of GAD65 immunotherapies, prevention of diabetes was associated with induction of Th2 regulatory T cells producing IL-4 and/or IL-10 (29, 30). Therefore, both $IL\text{-}4^{-/-}$ and $IL\text{-}10^{-/-}$ NOD mice were used to determine whether these cytokines are involved in agg Ig-GAD1-mediated protection against diabetes. Fig. 3 shows that treatment with agg Ig-GAD1 significantly delayed the disease in either strain of knockout mice. Thus, the mechanism by which agg Ig-GAD1 suppresses the disease does not seem to operate through IL-4 or IL-10 immune deviation (30).

Aging diminishes the effectiveness of Tregs against diabetes

Natural Tregs arise in the normal T cell repertoire to contribute to the maintenance of self-tolerance (1, 2). Gradual loss of function by Tregs is viewed as one of the lead mechanisms for development of autoimmunity in maturing NOD mice (7, 9). To address the issue of ineffectiveness of maturing Tregs, we began by examining whether treatment with agg Ig-GAD1 expanded Tregs in older mice positive for IAA, a sign indicative of insulitis (31, 32) and an ongoing disease process. Accordingly, mice were given agg Ig-GAD1 during the week of IAA seroconversion (which occurs at 8–11 wk of age) as well as 7 and 14 days later and then tested for expansion of Tregs. The results indicate that the number of $CD4^+CD25^+$ T cells had increased from 6.1% in the untreated mice to 10.1% in the age-matched, agg Ig-GAD1-treated animals (Fig. 4, *a* and *b*). However, when these animals were monitored for blood glucose levels, hyperglycemia was as prevalent as in the control untreated or agg Ig-HEL-treated groups, indicating a lack of protection against diabetes (Fig. 4*c*). In fact, the agg form of Ig-GAD1 had a similar result as the sol form, which is not effective in expanding Tregs (Fig. 4*c*). Overall, mice with progressive insulitis are able to expand Tregs, but fail to protect themselves against diabetes. Subsequently, the splenic $CD4^+CD25^+$ T cells from these mice were isolated and tested for suppression of passive diabetes mediated by pathogenic splenocytes of recently diabetic mice. These Tregs, however, were unable to protect the NOD.*scid* mice from diabetes; the survival pattern of the recipient mice was similar to that of animals given only the diabetogenic splenocytes (Fig. 4*d*). However, $CD4^+CD25^+$ T cells from the young NOD mice treated at 4, 5, and 6 wk of age were protective; 80% of the recipient animals were free of diabetes. It is thus logical to suspect that a decline in the suppressive function of Tregs is responsible for the lack of protection against the disease. To further address this matter, maturing natural and agg Ig-GAD1-expanded Tregs were isolated at different time points and tested for suppression of passive diabetes by cotransfer with diabetogenic splenocytes into NOD.*scid* mice. Fig. 5 shows that 70–80% of NOD.*scid* mice given young (6-wk-old) agg Ig-GAD1-expanded or natural (from untreated animals) Tregs remain free of diabetes (Fig. 5, *a* and *b*). The $CD4^+CD25^-$ counterparts had no significant effect on diabetes, and by wk 5 posttransfer, all animals became diabetic, as in the NOD.*scid* mice recipient of diabetogenic splenocytes without any Treg cotransfer. However, neither expanded nor natural Tregs taken at 8 wk of age (intermediate cells) could confer protection against the disease, and the incidence of diabetes was similar to that in animals that received the $CD4^+CD25^-$ counterparts (Fig. 5, *c* and *d*). Similarly, Tregs taken from protected 26-wk-old mice did not confer significant delay of diabetes relative to their $CD4^+CD25^-$ counterparts or the mice recipient of diabetogenic splenocytes without cotransfer (Fig. 5, *e* and *f*). Overall, these results indicate that Tregs abruptly lose their suppressive function at 8 wk of age and do not regain effectiveness by 26 wk of age.

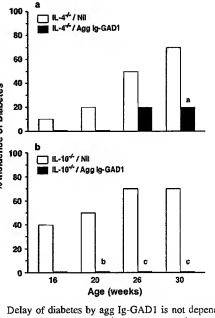

FIGURE 3. Delay of diabetes by agg Ig-GAD1 is not dependent upon IL-4 and IL-10. Groups of female $IL\text{-}4^{-/-}$ (*a*) and $IL\text{-}10^{-/-}$ (*b*) NOD mice (10/group) were given an i.p. injection of 300 μg of agg Ig-GAD1 (■) beginning at wk 4 through wk 12 and biweekly thereafter until 26 wk of age. The mice were monitored for blood glucose levels weekly up to wk 30 of age. Group of mice that did not receive any treatment with agg Ig-GAD1 (□) were included for control purposes. *a*, $p < 0.05$; *b*, $p<0.05$; *c*, $p<0.01$ (compared with nil groups).

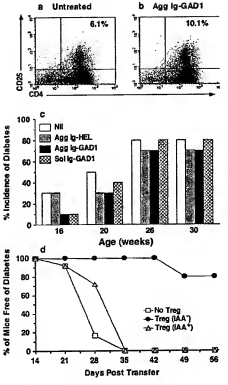

FIGURE 4. IAA-positive mice treated with agg Ig-GAD1 develop Tregs, but do not delay their diabetes. Groups of female NOD mice (10/group) positive for IAA were given an i.p. injection of 300 μg of agg Ig-GAD1 on the week of seroconversion as well as 7 and 14 days thereafter. The splenic cells from one untreated control group (*a*) as well as one treated group (*b*) were analyzed for CD4 and CD25 expression by flow cytometry 1 wk after the last injection. *c*, Groups of IAA-positive mice were given agg or sol chimeras according to the regimen described in *b*, and the mice were monitored for blood glucose levels up to wk 30 of age. A group of IAA-positive mice that did not receive any injection at any time (Nil) was included to serve as a control. *d*, Splenic $CD4^+CD25^+$ were isolated 3 days after completion of the treatment regimen, and 5×10^5 of these Tregs (IAA^-) were cotransferred with diabetogenic splenocytes into NOD.*scid* mice and tested for suppression of diabetes. For comparison purposes, NOD.*scid* mice recipient of diabetogenic splenocytes alone (No Treg) or together with Tregs isolated at the end of wk 6 from NOD mice treated with agg Ig-GAD1 at wk 4, 5, and 6 of age (IAA^-) were included.

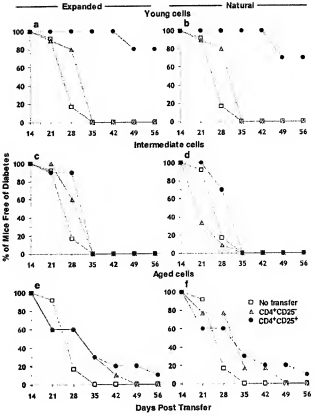

FIGURE 5. Young, but not intermediate or aged, Tregs suppress diabetes. Splenic $CD4^+CD25^+$ (●) and $CD4^+CD25^-$ (△) T cells from either untreated (Natural) or agg Ig-GAD1-treated (Expanded) mice were isolated at wk 6 (*a* and *b*; young), wk 8 (*c* and *d*; intermediate), or wk 26 (*e* and *f*; aged) of age. The cells were then coinjected i.v. with splenic cells derived from recently diabetic NOD females into recipient NOD.*scid* mice, and blood glucose levels were monitored every 7 days for a period of 56 days post-transfer. A group injected with diabetic splenocytes only (No transfer) was included for control purpose. Shown is the percentage of mice free of diabetes. These results are representative of two independent experiments.

Decline of mTGF-β expression on Tregs is responsible for loss of suppressive function

To investigate the mechanism underlying the loss of suppressive function, we began by ascertaining that the Tregs have not lost their phenotypic characteristics. Therefore, the intermediate (8-wk-old) Tregs were analyzed for CTLA-4 (33, 34), CD62L (35), and Foxp3 (27, 28) expression and compared with their young counterparts. It is shown that CTLA-4 expression on intermediate Tregs, whether expanded or natural, was similar to levels in young counterparts (Fig. 6, *a–c*). Similarly, CD62L expression was as significant on the intermediates as on the young Tregs (Fig. 6, *d–f*). Real-time PCR analysis revealed that Foxp3 mRNA expression was comparable in the intermediate Tregs vs their young counterparts (Fig. 6, *g* and *h*). Thus, the phenotypic characteristics of the Tregs were not altered over the transition from 6–8 wk of age.

It is therefore logical to suspect a defect in the function of these cells. Because these expanded Tregs do not secrete detectable levels of either IL-10 or TGF-β, it is likely that they conduct their suppressive function through physical contact (36). In recent years, mTGF-β has been suspected to be a major player in cell contact-mediated suppression by Tregs (10–13). Furthermore, dominant negative expression of TGF-β receptor type II on target effector $CD8^+$ Treg led to exacerbation of diabetes (14). The study then focused on analysis of surface expression of TGF-β on Tregs and its involvement in the functional discrepancy among young and intermediate Tregs. Accordingly, both young (6-wk-old) and intermediate (8-wk-old) expanded Tregs were assessed for cell mTGF-β. Fig. 7 shows that 39.0% of young expanded Tregs had mTGF-β. In contrast, only 12.1% of the intermediate population displayed mTGF-β (Fig. 7, *a* and *b*). Similar finding was observed with natural Tregs, as mTGF-β expression was reduced from 30.0% on the young Tregs to just 11.8% on their older counterparts (Fig. 7, *c* and *d*). Interestingly, Tregs from male NOD mice, which usually have lower incidence and delayed disease (37), did not decrease mTGF-β (Fig. 7, *e* and *f*). Indeed, 35.1% of the intermediate Tregs had significant mTGF-β, and this does not seem to reflect diminished expression, as 36.2% of the young Tregs also displayed mTGF-β. A decline in mTGF-β probably diminishes the suppressive function of Tregs. This statement emanates from the observation that blockade of mTGF-β abrogates the suppressive function of young Tregs both in vitro and in vivo (Fig. 7, *g* and *h*). In fact, young Tregs reduced the proliferation of their $CD4^+CD25^-$ counterparts to allogeneic cells. However, the

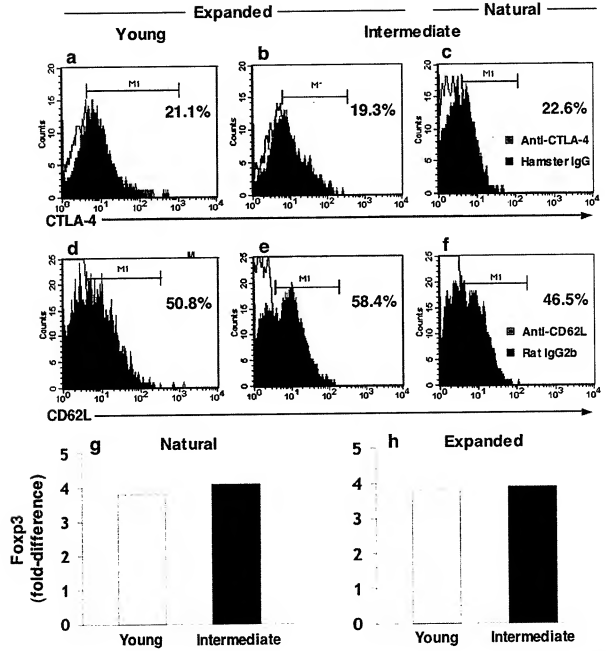

FIGURE 6. Young and intermediate Tregs display similar phenotypic patterns. Splenic $CD4^+$ T cells from either untreated (Natural) or agg Ig-GAD1-treated (Expanded) mice were isolated at 6 wk (*a* and *d*; young) or 8 wk (*b*, *c*, *e*, and *f*; intermediate) of age. CTLA-4 (*a–c*) and CD62L (*d–f*) cell surface expression was assessed on gated $CD4^+CD25^+$ T cells by flow cytometry. The marker, M1, represents the indicated percentage of cells positive for CTLA-4 or CD62L. *g* and *h*, Young and intermediate $CD4^+CD25^+$ T cells from untreated (Natural) and agg Ig-GAD-treated (Expanded) mice were isolated, and cytoplasmic RNA was used for analysis of Foxp3 mRNA expression by real-time PCR as described in *Materials and Methods*. The bars represent the fold increase in Foxp3 mRNA in $CD4^+CD25^+$ relative to the $CD4^+CD25^-$ counterpart.

alloreactivity of these effector cells was restored when the young Tregs were coated with anti-TGF-β Ab, but not isotype control mIgG (Fig. 7*g*). In vivo, when the young Tregs were coated with anti-TGF-β Ab before cotransfer with diabetogenic splenocytes, the recipient NOD.*scid* mice developed diabetes (Fig. 7*h*). However, coating with mIgG instead of anti-TGF-β sustained resistance against diabetes. Therefore, young NOD Tregs require the activity of mTGF-β to effectively suppress the function of effector cells.

Taken together, these data indicate that an abrupt age-dependent loss of mTGF-β on Tregs lessens immune regulation of effector cells, leading to the onset of destructive insulitis and progression to diabetes.

Discussion

Ig-GAD1 expressing aa sequence 524–543 of GAD65 expands Tregs upon administration to NOD mice in an agg form (Fig. 1). These Tregs display significant suppressive functions against effector cells despite the lack of detectable secretion of IL-10 or TGF-β. Treatment with agg Ig-GAD1 at the age of 4 wk reduced the spontaneous proliferative T cell responses that usually develop

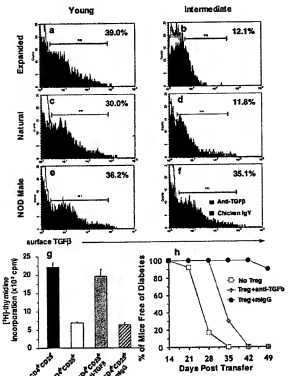

FIGURE 7. A sudden decline in mTGF-β expression is responsible for the lack of effectiveness of intermediate Treg against diabetes. Splenic $CD4^+$ T cells were isolated from agg Ig-GAD1-treated (*a* and *b*; expanded) or untreated (*c* and *d*; natural) mice at 6 wk (*a* and *c*; young) and 8 wk (*b* and *d*; intermediate) of age. The cells were then tested for cell surface expression of TGF-β by flow cytometry. For comparison purposes, $CD4^+$ T cells from untreated NOD male mice were also isolated at 6 wk (*e*) and 8 wk (*f*) of age and tested for surface TGF-β. The histograms were gated on double-positive $CD4^+CD25^+$ T cells. *g*, Agg Ig-GAD1-expanded young $CD4^+CD25^+$ T cells were tested in vitro for suppression of their $CD4^+CD25^-$ T counterparts in the presence of anti-TGF-β Ab using the allogeneic proliferation system described in Fig. 1. The $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were used at a 1:1 ratio (200×10^3 cells/well for each type). The $CD4^+CD25^+$ T cells were precoated for 2 h with 100 μg/ml anti-TGF-β Ab or mouse IgG isotype control before addition of allogeneic and target $CD4^+CD25^-$ T cells. Each bar represents the mean $\pm$ SD of triplicate wells. *h*, Agg Ig-GAD1-expanded young splenic $CD4^+CD25^+$ T cells (500×10^3 cells/mouse) were precoated with anti-TGF-β (Treg+anti-TGF-β) or mouse IgG isotype control (Treg+migG), then coinjected i.v. with diabetogenic splenocytes into NOD.*scid* mice. Blood glucose levels were monitored weekly. A recipient group injected with diabetic splenocytes only (No Treg) was included as a control.

in NOD mice and delayed diabetes (Fig. 2). Depletion of Tregs during administration of agg Ig-GAD1 resulted in a lack of protection against diabetes. Moreover, titration of Tregs in the protected animals indicated maintenance of the elevated frequency for the 30-wk period of clinical monitoring. Therefore, it appears that agg Ig-GAD1-mediated delay of diabetes is dependent on Treg function. In fact, DC-expanded specific Tregs proved potent against diabetes (38). In addition, it seems likely that suppression of diabetogenic T cells by the Tregs is mediated by cell contact, rather than cytokines. This statement stems from the observations that Tregs were unable to secrete TGF-β or IL-10, and treatment with Ig-GAD1 was effective against diabetes in both $IL\text{-}10^{-/-}$ and $IL\text{-}4^{-/-}$ mice (Fig. 3). Surprisingly, however, treatment with agg Ig-GAD1 was not effective against diabetes in 8-wk-old, IAA-positive mice despite expansion of Tregs (Fig. 4). Functional analysis of these mature cells indicated an inability to suppress passive diabetes, whereas counterparts expanded before IAA seroconversion protected against the disease. These findings, although puzzling, suggested that young Tregs, which protect against the disease and maintain a significant frequency thereafter, lose their suppressive functions over time and become unable to oppose the disease. In an attempt to explore this postulate, maturing Tregs were isolated at different ages and tested for protection against diabetes. The findings indicated that 6-wk-old Tregs, which we refer to as young Tregs, are endowed with suppressive functions and protect against passive diabetes (Fig. 5). These results agree with reports showing that young Tregs protect against aggressive diabetes mediated by islet diabetogenic T cells for even a longer period of time (35). In contrast, 8- and 26-wk-old Tregs, which we refer to as intermediate and aged Tregs, respectively, were unable to confer resistance against the disease (Fig. 5). The fact that natural Tregs do not decline in number over time in naive untreated mice (Fig. 2) and protect against diabetes when tested as young, rather than intermediate or aged Tregs (Fig. 5), again indicates a time-sensitive loss of function.

Analysis of the expression of Treg markers on these nonprotective cells showed an unaffected phenotype, because CTLA-4, CD62L, and Foxp3 expressions were similar on young protective and older nonprotective Tregs, whether natural or expanded by agg Ig-GAD1 (Fig. 6). In the face of this dilemma, we resorted to exploring any involvement of mTGF-β in this loss of function by Tregs. Surprisingly, the young Tregs expressed significant levels of active mTGF-β, but over a transition period of 2–3 wk, during which IAA seroconversion took place and an abrupt decline in mTGF-β expression transpired, persisting up to wk 26 of age (Fig. 7). This decline was not observed with Tregs of male NOD mice and thus concurs with the protection observed with aged male Tregs (6). Interestingly, blockade of mTGF-β by anti-TGF-β Ab abolished the suppressive function of young Tregs, leading to a lack of protection against diabetes. These findings indicate that a decline in mTGF-β during the transition to IAA seroconversion nullifies the suppressive function of Tregs. Thus, although the cells remain expandable by agg Ig-GAD1 and maintain a significant frequency, an abrupt loss of mTGF-β during maturation drives a loss of function and a lack of protection against diabetes. These results suggest that Tregs are able to suppress pathogenic T cells up to wk 8 of age, then a loss of mTGF-β occurs, which nullifies their suppressive function, leading to a lack of protection at later stages of the disease. It should be noted, however, that this Treg functional impairment would not affect protected animals, because their pathogenic T cells have already been down-regulated. Given that mTGF-β has been implicated in Treg function (10–13), the age-dependent decline in its expression bodes well with the report describing a loss of function by Tregs at 16 wk of age (9). Also, this would provide a mechanism for circumstances under which disease eruption occurs despite the presence of an unaltered frequency of Tregs (6, 7). The abrupt transition for loss of function at 8 wk of age may be critical for massive release of diabetogenic cells from suppression to ensure perpetuation of the 6- to 8-wk-long destructive insulitis and resultant onset of diabetes (39). Although, this observation sheds light on the loss of function by Tregs operating suppression through mTGF-β, other mechanisms may be in place for cells operating through cell surface expression of GITR (40, 41), production of IL-10 (42, 43), or secreted TGF-β (44, 45). In fact, we found that Ig-INSβ, a chimera expressing $INS\beta_{9-23}$ peptide expands Tregs that produce IL-10 and protects young animals against diabetes (43). However, at later stages of the disease when the diabetogenic T cells reach the islets and become activated, IL-10 down-regulates their CTLA-4, thus hindering the CTLA-4 inhibitory pathway, to sustain T

cell activation and nullify the protective function of Tregs (43). Compensatory mechanisms seem to be available, however, because stimulation with anti-CD3 Ab at later stages of the disease mobilizes Tregs that secrete TGF-β and protects against the disease (44). Finally, these findings shed light on the efficacy of Ag- and cytokine-based approaches against diabetes at early, but not later, stages of the disease.

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References

1. Shevach, E. M. 2002. CD4$^+$ CD25$^+$ suppressor T cells: more questions than answers. *Nat. Rev. Immunol. 2:389.*
2. Sakaguchi, S. 2004. Naturally arising CD4$^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu. Rev. Immunol. 22:531.*
3. von Herrath, M. G., and L. L. Harrison. 2003. Antigen-induced regulatory T cells in autoimmunity. *Nat. Rev. Immunol. 3:223.*
4. Yamazaki, S., T. Iyoda, K. Tarbell, K. Olson, K., Velinzon, K. Inaba, and R. M. Steinman. 2003. Direct expansion of functional CD25$^+$ CD4$^+$ regulatory T cells by antigen processing dendritic cells. *J. Exp. Med. 198:235.*
5. Walker, L. S. K., A. Chodos, and A. K. Abbas. 2003. Antigen-dependent proliferation of CD4$^+$ CD25$^+$ regulatory T cells in vivo. *J. Exp. Med. 198:249.*
6. Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4$^+$ CD25$^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity 12:431.*
7. Delovitch, T. L., and B. Singh. 1997. The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity 7:727.*
8. Wu, A. J., H. Hua, S. H. Munson, and H. O. McDevitt. 2002. Tumor necrosis factor-α regulation of CD4$^+$CD25$^+$ T cell levels in NOD mice. *Proc. Natl. Acad. Sci. USA 99:12287.*
9. Gregori, S., N. Gherardana, S. Smiroldo, and L. Adorini. 2003. Dynamics of pathogenic and suppressor T cells in autoimmune diabetes development. *J. Immunol. 171:4040.*
10. Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by CD4$^+$CD25$^+$ regulatory T cells is mediated by cell surface-bound transforming growth factor β. *J. Exp. Med. 194:629.*
11. Nakamura, K., A. Kitani, I. Fuss, A. Pedersen, N. Harada, H. Nawata, and W. Strober. 2004. TGF-β1 plays an important role in the mechanism of CD4$^+$CD25$^+$ regulatory T cell activity in both humans and mice. *J. Immunol. 172:834.*
12. Chen, W., W. Jin, N. Hardegen, K. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4$^+$CD25$^-$ naive T cells to CD4$^+$CD25$^+$ regulatory T cells by TGF-β induction of transcription factor *foxp3. J. Exp. Med. 198:1875.*
13. Oida, T., X. Zhang, M. Goto, S. Hachimura, M. Totsuka, S. Kaminogawa, and H. L. Weiner. 2003. CD4$^+$CD25$^-$ T cells that express latency-associated peptide on the surface suppress CD4$^+$CD45RB^{high}-induced colitis by a TGF-β dependent mechanism. *J. Immunol. 170:2516.*
14. Green, E. A., L. Gorelik, C. M. McGregor, E. H. Tran, and R. A. Flavell. 2003. CD4$^+$CD25$^+$ T regulatory cells control anti-islet CD8$^+$ T cells through TGF-β-TGF-β receptor interactions in type 1 diabetes. *Proc. Natl. Acad. Sci. USA 100:10878.*
15. Quinn, A., B. McInerney, E. P. Reich, O. Kim, K. P. Jensen, and E. E. Sercarz. 2001. Regulatory and effector CD4$^+$ T cells in non-obese diabetic mice recognize overlapping determinants of glutamic acid decarboxylase and use distinct Vβ genes. *J. Immunol. 166:2982.*
16. Wilson, S. S., T. C. White, and D. DeLuca. 2001. Therapeutic alteration of insulin-dependent diabetes mellitus progression by T cell tolerance to glutamic acid decarboxylase 65 peptides in vitro and in vivo. *J. Immunol. 167:569.*
17. Wang, B., A. Gonzalez, P. Hoglund, J. D. Katz, C. Benoist, and D. Mathis. 1998. Interleukin-4 deficiency does not exacerbate disease in NOD mice. *Diabetes 47:1207.*
18. Serreze, D. V., H. D. Chapman, C. M. Post, B. A. Johnson, W. L. Suarez-Pinzon, and A. Rabinovitch. 2001. Th1 to Th2 cytokine shifts in nonobese diabetic mice: sometimes an outcome, rather than the cause of diabetes resistance elicited by immunostimulation. *J. Immunol. 166:1352.*
19. Daniel, D., and D. R. Wegmann. 1996. Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B (9–23). *Proc. Natl. Acad. Sci. USA 93:956.*
20. Kaufman, D. L., M. Clare-Salzler, J. Tian, T. Forsthuber, G. S. Ting, P. Robinson, M. A. Atkinson, E. B. Sercarz, A. J. Tobin, and P. V. Lehmann. 1993. Spontaneous loss of T cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature 366:69.*
21. Tisch, R., X. D. Yang, S. M. Singer, R. S. Liblau, L. Fugger, and H. O. McDevitt. 1993. Immune response to glutamic acid decarboxylase correlates with insulitis in nonobese diabetic mice. *Nature 366:72.*
22. Chao, C.-C., H.-K. Sytwu, E. L. Chen, J. Toma, and H. O. McDevitt. 1999. The role of MHC class II molecules in susceptibility to type 1 diabetes: identification of peptide epitopes and characterization of the T cell repertoire. *Proc. Natl. Acad. Sci. USA 96:9299.*
23. Latek, R. R., A. Suri, S. J. Petzold, C. A. Nelson, O. Kanagawa, E. R. Unanue, and D. H. Fremont. 2000. Structural basis of peptide binding and presentation by the diabetes-associated MHC class II molecule of NOD mice. *Immunity 12:699.*
24. Legge, K. L., B. Min, J. J. Bell, J. C. Caprio, L. Li, R. K. Gregg, and H. Zaghouani. 2000. Coupling of peripheral tolerance to endogenous interleukin 10 promotes effective modulation of myelin-activated T cells and ameliorates experimental allergic encephalomyelitis. *J. Exp. Med. 191:2039.*
25. Legge, K. L., J. J. Bell, L. Li, R. K. Gregg, J. C. Caprio, and H. Zaghouani. 2001. Multi-modal antigen specific therapy for autoimmunity. *Int. Rev. Immunol. 20:593.*
26. Legge, K. L., R. K. Gregg, R. Maldonado-Lopez, L. Li, J. C. Caprio, M. Moser, and H. Zaghouani. 2002. On the role of dendritic cells in peripheral T cell tolerance and modulation of autoimmunity. *J. Exp. Med. 196:217.*
27. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. *Nat. Immunol. 4:330.*
28. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science 299:1057.*
29. Tisch, R., R. S. Liblau, X. D. Yang, R. Liblau, and H. O. McDevitt. 1998. Induction of GAD65 specific regulatory T-cells inhibits ongoing autoimmune diabetes in nonobese diabetic mice. *Diabetes 47:894.*
30. Tian, J., M. Clare-Salzler, A. Herschenfeld, B. Middleton, D. Newman, R. Mueller, S. Arita, C. Evans, M. A. Atkinson, Y. Mullen, et al. 1996. Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. *Nat. Med. 2:1348.*
31. Robles, D. T., G. S. Eisenbarth, N. J. M. Dailey, L. B. Peterson, and L. Wicker. 2003. Insulin autoantibodies are associated with islet inflammation but not always related to diabetes progression in NOD congenic mice. *Diabetes 52:882.*
32. Yu, L., D. T. Robles, N. Abiru, P. Kaur, M. Rewers, K. Kelemen, and G. S. Eisenbarth. 2000. Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes. *Proc. Natl. Acad. Sci. USA 97:1701.*
33. Read, S., V. Malmstron, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen-4 plays an essential role in the function of CD25$^+$CD4$^+$ regulatory cells that control intestinal inflammation. *J. Exp. Med. 192:295.*
34. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by CD25$^+$CD4$^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med. 192:303.*
35. Szanya, V., J. Ermann, C. Taylor, C. Holness, and C. G. Fathman. 2002. The subpopulation of CD4$^+$CD25$^+$ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. *J. Immunol. 169:2461.*
36. Thornton, A. M., and E. M. Shevach. 1998. CD4$^+$CD25$^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med. 188:287.*
37. Adorini, L., S. Gregori, and L. C. Harrison. 2002. Understanding autoimmune diabetes: insights from mouse models. *Trends Mol. Med. 2:31.*
38. Tarbell, K. V., S. Yamazaki, K. Olson, P. Toy, and R. M. Steinman. 2004. CD25$^+$ CD4$^+$ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. *J. Exp. Med. 199:1467.*
39. André, I., Gonzalez, A., Wang, B., Katz, J. D., Benoist, C., and Mathis, D. 1996. Checkpoints in the progression of autoimmune disease: Lessons from diabetes models. *Proc. Natl. Acad. Sci. USA 93:2260.*
40. Shimizu, J., S. Yamazaki, T. Takahashi, Y. Ishida, and S. Sakaguchi. 2001. Stimulation of CD25$^+$CD4$^+$ regulatory T cells through GITR breaks immunological self-tolerance. *Nat. Immunol. 3:135.*
41. McHugh, R. S., M. J. Whitters, C. A. Piccirillo, D. A. Young, E. M. Shevach, M. Collins, and M. C. Byrne. 2002. CD4$^+$CD25$^+$ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity 16:311.*
42. Maloy, K. J., L. Salaun, R. Cahill, G. Dougan, N. J. Saunders, and F. Powrie. 2003. CD4$^+$CD25$^+$ T_R cells suppress innate immune pathology through cytokine-dependent mechanisms. *J. Exp. Med. 197:111.*
43. Gregg, R. K., J. J. Bell, H.-H. Lee, R. Jain, S. J. Schoenleber, R. Divekar, and H. Zaghouani. IL-10 diminishes CTLA-4 expression on islet-resident T cells and sustains their activation rather than tolerance. *J. Immunol. In press.*
44. Belghith, M., J. A. Bluestone, S. Barriot, J. Megret, J. Bach, and L. Chatenoud. 2003. TGFβ-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat. Med. 9:1202.*
45. Peng, Y., Y. Laouar, M. O. Li, A. G. Green, and R. A. Flavell. 2004. TGF-β regulates in vivo expansion of Foxp3-expressing CD4$^+$CD25$^+$ regulatory T cells responsible for protection against diabetes. *Proc. Natl. Acad. Sci. USA 101:4572.*

IL-10 Diminishes CTLA-4 Expression on Islet-Resident T Cells and Sustains Their Activation Rather Than Tolerance[1]

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IL-10, a powerful anti-Th1 cytokine, has shown paradoxical effects against diabetes. The mechanism underlying such variable function remains largely undefined. An approach for controlled mobilization of endogenous IL-10 was applied to the NOD mouse and indicated that IL-10 encounter with diabetogenic T cells within the islets sustains activation, while encounter occurring peripheral to the islets induces tolerance. Insulin β-chain (INSβ) 9-23 peptide was expressed on an Ig, and the aggregated (agg) form of the resulting Ig-INSβ triggered IL-10 production by APCs, and expanded IL-10-producing T regulatory cells. Consequently, agg Ig-INSβ delayed diabetes effectively in young NOD mice whose pathogenic T cells remain peripheral to the islets. However, agg Ig-INSβ was unable to suppress the disease in 10-wk-old insulitis-positive animals whose diabetogenic T cells have populated the islets. This is not due to irreversibility of the disease because soluble Ig-INSβ did delay diabetes in these older mice. Evidence is provided indicating that upon migration to the islet, T cells were activated and up-regulated CTLA-4 expression. IL-10, however, reverses such up-regulation, abolishing CTLA-4-inhibitory functions and sustaining activation of the islet T lymphocytes. Therefore, IL-10 supports T cell tolerance in the periphery, but its interplay with CTLA-4 sustains activation within the islets. As a result, IL-10 displays opposite functions against diabetes in young vs older insulitis-positive mice. *The Journal of Immunology*, 2005, 174: 662–670.

Type 1 or insulin-dependent diabetes mellitus (IDDM)[4] is regarded as an immune-mediated disease in which the β cells of the pancreatic islets of Langerhans are destroyed as a consequence of inflammatory reactions triggered by activation of T cells specific for β cell-associated Ags (1, 2). The NOD mouse develops spontaneous diabetes that shares many of the features associated with human IDDM, providing a well-characterized animal model for this complex autoimmune disease (3). In the NOD mouse model, like in human IDDM, self-reactive Th1 cells play a major role in the initial stages of the disease (4). IL-10, a powerful anti-Th1 cytokine, has in recent years shown variable effects against type 1 diabetes (5–9). The mode of delivery of the cytokine (5–7) as well as the age of the animals (8, 9) are believed to be contributing factors to the erratic behavior of IL-10. The question then is how IL-10 in the blood affects diabetes differently from IL-10 expressed in the islet. Also, how does IL-10 suppress diabetes in young animals whose diabetogenic T cells remain peripheral to the islets, but display no effectiveness in older animals whose diabetogenic T cells are spread both in the periphery and the islets? One potential response to these questions is that peripheral and islet-resident diabetogenic T cells display differential susceptibility to regulation by IL-10. The studies presented in this work devised a unique strategy for mobilizing and targeting endogenous IL-10 to diabetogenic T cells and attempted to explore this postulate.

We have previously shown that expression of myelin peptides on Ig facilitates internalization through FcγR and increases peptide presentation to T cells (10). In addition, aggregation of the Ig-myelin chimera, which cross-links FcγR, induced IL-10 production by APCs and sustained effective suppression of experimental allergic encephalomyelitis (11–13). Recently, IL-10 has been shown to serve as a growth factor for T regulatory (Treg) cells (14, 15). In fact, in vitro (16) as well as in vivo (17) regimens using IL-10 successfully induced Treg cells that produce IL-10 and support tolerance against pathogenic T cells.

In this study, the I-A^{g7}-restricted insulin β-chain (INSβ) 9-23 peptide (18, 19) was genetically engineered into the V region of an Ig molecule, and the resulting Ig-INSβ was aggregated (agg) and tested for induction of IL-10-producing Treg cells and suppression of diabetes. Both young NOD mice that have not progressed to insulitis and older animals positive for insulin autoantibody (IAA), which is indicative of insulitis, were included in the studies. The results indicate that agg Ig-INSβ induced IL-10 production by APCs and sustained the development of IL-10-producing Treg cells in vivo. Moreover, when given to 4-wk-old NOD mice, agg Ig-INSβ suppressed diabetogenic T cells and protected the mice against diabetes. This effect is most likely due to down-regulation by IL-10 from APCs and/or Treg cells because: 1) soluble (sol) Ig-INSβ, not inducing IL-10, was less effective against the disease; 2) agg Ig-INSβ was unable to protect young IL-10-deficient mice from diabetes; and 3) depletion of Treg cells at young age also

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[4] Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; agg, aggregated; DC, dendritic cell; *Foxp3*, forkhead/winged helix transcription factor gene; GAD, glutamic acid decarboxylase; HEL, hen egg lysozyme; IAA, insulin autoantibody; INSβ, insulin β-chain; sol, soluble; Treg, T regulatory.

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hinders agg Ig-INSβ-mediated delay of the disease. Surprisingly, however, agg Ig-INSβ was unable to delay diabetes in IAA-positive mice despite the fact that the disease remained reversible as the sol form of Ig-INSβ was able to reverse it. Evidence is provided indicating that T cells up-regulate CTLA-4 upon migration to the islets and agg Ig-INSβ reversed such expression both in vitro and in vivo through APC and/or Treg cell-derived IL-10. The end result is sustained activation of the diabetogenic T cells. Given the fact that IAA-positive IL-$10^{-/-}$ mice were able to reverse their diabetes upon treatment with agg Ig-INSβ, it is suggested that down-regulation of CTLA-4 by IL-10 nullifies its inhibitory functions and sustains T cell activation and lack of protection against diabetes.

Materials and Methods

Mice

NOD ($H\text{-}2^{g7}$) and NOD.scid mice were purchased from The Jackson Laboratory, and IL-10-deficient ($IL\text{-}10^{-/-}$) NOD mice were previously described (20). The experimental procedures performed on these animals were conducted according to the guidelines of the institutional animal care and use committee.

Assessment of diabetes

Mice are bled from the tail vein weekly, and the blood samples are used to assess glucose content using test strips and an Accu-Chek Advantage monitoring system (Roche Diagnostics). A mouse is considered diabetic when the blood glucose is above 300 mg/dl for 2 consecutive wk.

Antigens

Peptides. All peptides used in this study were purchased from Metabion and purified by HPLC to >90% purity. INSβ peptide encompasses the diabetogenic INSβ 9-23 amino acid sequence (SHLVEALYLVCGERG). Glutamic acid decarboxylase 2 (GAD2) peptide corresponds to aa residues 206–220 (TVEIAPVFVLLEYVT) of GAD65 (21). Hen egg lysozyme (HEL) peptide encompasses a nondiabetogenic epitope corresponding to aa residues 11–25 (AMKRHGLDNYRGYSL) of HEL (22). INSβ, GAD2, and HEL peptides are presented to T cells in association with $I\text{-}A^{g7}$ MHC class II molecules.

Ig chimeras. Ig-INSβ, Ig-GAD2, and Ig-HEL express INSβ, GAD2, and HEL peptide, respectively. Insertion of INSβ, GAD2, and HEL nucleotide sequences into the CDR3 of the H chain V region of the 91A3 IgG2b, κ Ig was conducted as described (10). Large-scale cultures of transfectoma cells were conducted in DMEM medium containing 10% iron-enriched calf serum (BioWhittaker). Purification of the chimeras was conducted on separate columns of rat anti-mouse κ-chain mAb coupled to CNBr-activated 4B Sepharose (Amersham Biosciences). Aggregation of the Ig chimeras was conducted by precipitation with 50% saturated $(NH_4)_2SO_4$, as described (11).

T cell lines

T cell lines specific for INSβ, GAD2, and HEL peptides were generated by immunizing NOD mice s.c. with 100 μg of peptide in CFA and in vitro peptide stimulation, followed by resting, as described (11). These lines are of Th1-type T cells and produce IFN-γ, but not IL-4 or IL-10 upon stimulation with the corresponding peptide or sol Ig chimera (data not shown).

Isolation of T cells

CD4 and CD8 T lymphocytes were isolated from splenic or islet cells by positive selection on Miltenyi (Miltenyi Biotec) microbeads, according to the manufacturer's instructions. Isolation of islet CD4 and CD8 T cells was performed, as described (23). Isolation of splenic $CD4^+CD25^+$ T cells was conducted by negative selection of CD4 T cells, followed by positive selection by anti-CD25 Ab coupled to microbeads, according to Miltenyi's instructions.

Isolation of APCs

Splenic dendritic cells (DC) were purified, according to a standard collagenase/differential adherence method (13). Briefly, the spleen was disrupted in a collagenase solution, and isolated DC floated on a dense BSA gradient. Subsequently, the cells were allowed to adhere to petri dishes for 90 min at 37°C, washed, and incubated overnight. The DC were then harvested and further purified on anti-CD11c-coupled microbeads, according to Miltenyi's instructions. Partial purification of splenic APCs was conducted by floating the cells on a dense BSA gradient as for the DC, and the cells were washed in plain culture medium and used in presentation assays. These APCs are designated BSA-APCs.

Flow cytometry analyses

For staining of $CD4^+CD25^+$ T cells, purified splenic CD4 T cells (1×10^6 cells/ml) were incubated with anti-CD4-FITC and anti-CD25-APC or isotype control rat IgG1 APC for 30 min at 4°C and washed with buffer. The cells were then fixed with 2% formaldehyde for 20 min at 25°C and then analyzed. Events ($30–50 \times 10^3$) were collected on a FACSVantage flow cytometer (BD Biosciences) and analyzed using CellQuest software 3.3 (BD Biosciences). Staining for CTLA-4 was conducted, as follows: purified islet and splenic CD4 T cells (1×10^6 cells/ml) were incubated with anti-CTLA-4-PE or isotype control hamster IgG1 for 2 h at 37°C, followed by anti-CD4-FITC for 30 min at 4°C. The cells were then washed, fixed with 2% formaldehyde, and analyzed on a FACSVantage flow cytometer, as above.

Proliferation assays

For T cell line proliferation assay, irradiated (3000 rad) NOD female splenocytes (5×10^5 cells/50 μl/well) were incubated with graded amounts of either Ig-INSβ or Ig-HEL (100 μl/well), and 1 h later INSβ-specific T cells (5×10^4 cells/well/50 μl) were added. After 72 h, 1 μCi of [^{3}H]thymidine was added per well, and the culture was continued for an additional 14.5 h. The cells were then harvested on a Tritux 1450 Microbeta Wallac Harvester, and Incorporated [^{3}H]thymidine was counted using the Microbeta 270.004 software (EG & G Wallac).

For evaluation of T cell responses in vivo, purified splenic CD4 T cells (2.5×10^5 cell/well) isolated from 16-wk-old untreated or agg Ig-INSβ-treated mice were stimulated with irradiated (3000 rad) BSA-APCs (5×10^5 cells/well) and 30 μg/ml peptide, and proliferation was measured, as above.

For proliferation of Treg cells, purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells (2×10^5 cells/well) were incubated for 72 h with 18 μM INSβ or HEL and irradiated (3000 rad) BSA-APCs (4×10^5 cells/well), and proliferation was assessed

T cell cytokine assays

All cytokine analyses were done by ELISA using anti-cytokine Abs from BD Pharmingen, as described (13).

Analysis of the effect of APC's IL-10 on IFN-γ production was done as follows: the T cell line (0.2×10^5 cells/well) was incubated with purified NOD splenic DC (5×10^4 cells/well) and Ig chimeras for 24 h, and IFN-γ as well as IL-10 were measured by ELISA. In some experiments, blockade of IL-10 was performed by supplementing the culture with 40 μg/ml anti-IL-10 Ab JES5-2A5. The Isotype control used 40 μg/ml rat IgG.

For evaluation of cytokine T cell responses in vivo, purified splenic CD4 T cells (2.5×10^5 cell/well) isolated from 16-wk-old untreated or agg Ig-INSβ-treated mice were stimulated with irradiated (3000 rad) BSA-APC (5×10^5 cells/well), and 30 μg/ml peptide and IFN-γ as well as IL-10 were measured by ELISA after 48-h Incubation.

For assessment of IL-10 production by Treg cells, purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells (2×10^5 cells/well) were incubated for 48 h with 10 μg/ml plate-bound anti-CD3 Ab (2C11), and the cytokine was measured by ELISA.

For evaluation of IFN-γ production by islet-resident T cells, bulk islet cells (5×10^3 cells/well) were stimulated with 18 μM INSβ peptide or 1 μM Ig chimeras for 48 h, and IFN-γ was measured by ELISA. In the case of purified islet lymphocytes, the CD4 or CD8 T cells (2×10^5 cells/well) were incubated with irradiated BSA-APCs (5×10^5 cells/well) and 1 μM Ig chimeras. IFN-γ was measured 48 h later by ELISA.

RT-PCR for Foxp3 expression

Total RNA was extracted from cells using TRIzol reagent and used to determine relative mRNA levels of forkhead/winged helix transcription factor gene (*Foxp3*). Reverse transcription and DNA amplification were performed using 300 ng of total RNA, 100 ng of Foxp3 and β-actin primers (24), and the QuantiTect SYBR Green RT-PCR kit from Qiagen, as described (25).

Adoptive transfer

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified from the spleen of 6-wk-old agg Ig-INSβ-treated mice, and 5×10^5 cells were cotransferred i.v. with 10×10^6 diabetic splenocytes into NOD.scid mice (4–8 wk of age). The animals were monitored for blood glucose levels weekly.

Depletion of Treg cells

For depletion of $CD25^+$ T cells in vivo, NOD mice were given 1 mg/mouse anti-CD25 mAb (PC61) alone or concurrently with agg Ig-INSβ injection. Rat IgG (1 mg/mouse) was used as a control.

Detection of IAA

The following was conducted by ELISA: microtiter plates were coated with 50 μl of sodium bicarbonate solution (pH 9.6) containing 10 μg/ml porcine insulin (Sigma-Aldrich) for 16 h at 4°C. The plates were then washed three times with PBS-0.05% Tween 20 and saturated with 2.5% ossein (in 0.3 M NaCl, pH 7) for 2 h. Serum samples (1/200 dilutions) were then added for 16 h at 4°C, followed by biotin-conjugated rat anti-mouse κ mAb (100 μl at 1 μg/ml). The plates were then incubated with avidin peroxidase (2.5 mg/ml) for 30 min at 25°C, and the assay was revealed by addition of ABTS substrate. The samples were read at 405 nm on a Spectramax 190. A sample is considered IAA positive when the OD_{405} is >0.2. This cutoff line of 0.2 was chosen because serum samples from 10 SIL mice, which are nonprone to diabetes development and presumably do not produce insulin-specific autoantibodies, never exceeded 0.05 OD_{405} (4-fold less than cutoff).

Statistical analysis

The χ^2 test was used for data analysis among experimental and control groups. Cytokine levels were compared using Student's *t* test for unpaired samples.

Results

Agg Ig-INSβ triggers IL-10 production by APCs and supports the development of Treg cells

Recent studies have revealed that delivery of myelin peptides on Igs enhances presentation to T cells (12). Moreover, aggregation of Ig-myelin chimeras induced IL-10 production by APCs and sustained effective down-regulation of myelin-reactive T cells (11, 13). Because IL-10 can serve as a growth factor for Treg cells (14), delivery of self peptides on IL-10-inducing agg Igs could support the development of Treg cells and sustain additive tolerogenic functions that should be effective against complex autoimmunity such as type 1 diabetes. To test this premise, the $I\text{-}A^{g7}$-restricted diabetogenic INSβ peptide was expressed on an Ig and the resulting Ig-INSβ chimera was tested for presentation to INSβ-specific T cells, triggering of IL-10 production by APCs, and induction of Treg cells. Fig. 1*A* shows that Ig-INSβ, but not the control Ig-HEL, induced significant proliferation of INSβ-specific T cells. These results indicate that Ig-INSβ was taken up by the APCs and processed, and an INSβ peptide was generated and presented to T cells. Also, agg, but not sol Ig-INSβ induced IL-10 production by DC (Fig. 1*B*). As IL-10 can serve as a growth factor for Treg cells (14, 15), treatment with agg Ig-INSβ may support the development of Treg cells in vivo. Fig. 1, *C* and *D*, shows that agg Ig-INSβ increased $CD4^+CD25^+$ T cells from 4.4% in untreated to 7.1% in agg Ig-INSβ-treated nondiabetic NOD mice. Moreover, these $CD4^+CD25^+$ T cells had increased *Foxp3* mRNA expression relative to their $CD4^+CD25^-$ counterparts (Fig. 1*E*), but displayed reduced proliferation upon stimulation with INSβ peptide (Fig. 1*F*). $CD4^+CD25^+$ T cells from untreated mice also had 4-fold higher Foxp3 expression (data not shown). Interestingly, stimulation with anti-CD3 Ab induced increased IL-10 production by the expanded relative to the natural $CD4^+CD25^+$ T cells or the $CD4^+CD25^-$ counterparts (Fig. 1*G*). The lack of increased IL-10 production by the natural $CD4^+CD25^+$ T cells may be related to lower frequency of IL-10-producing cells among this heterogeneous population, while treatment with agg Ig-INSβ specifically expands IL-10-producing T cells. Finally, upon transfer to NOD.scid mice, the $CD4^+CD25^+$, but not $CD4^+CD25^-$ T cells conferred protection against passive diabetes mediated by diabetogenic splenocytes (Fig. 1*H*). Thus, these $CD4^+CD25^+$ T cells represent Tregs rather than activated $CD4^+$ T cells because they have higher Foxp3 expression relative to their $CD4^+CD25^-$ counterparts as did $CD4^+CD25^+$ T cells from untreated mice, were not proliferative upon stimulation with INSβ peptide, and suppressed diabetes upon transfer into NOD.scid mice along with pathogenic splenocytes. Overall, these results indicate that agg Ig-INSβ supports the development of IL-10-producing Treg cells endowed with suppressive functions.

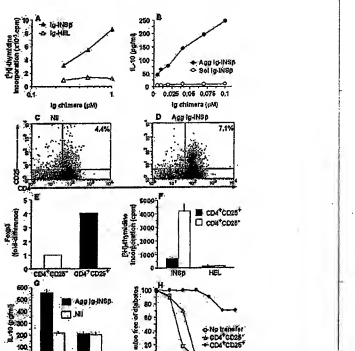

FIGURE 1. Agg Ig-INSβ expands IL-10-producing Treg cells. *A*, Presentation of Ig-INSβ and the control Ig-HEL to INSβ-specific T cells by irradiated splenic NOD APCs was assessed by $[^3H]$thymidine incorporation. *B*, The ability of agg and sol Ig-INSβ to induce IL-10 production by DC was measured by incubating 100×10^3 purified DC and measuring the cytokine 24 h later by ELISA. Each point represents the mean of triplicate wells. Detection of splenic $CD4^+CD25^+$ T cells from 16-wk-old NOD mice untreated (*C*) or treated (*D*) with agg Ig-INSβ at weeks 4, 5, and 6 of age was assessed by flow cytometry. *Foxp3* mRNA expression (*E*) and proliferation (*F*) of $CD4^+CD25^+$ T cells in comparison with their $CD4^+CD25^-$ counterparts were analyzed by real-time PCR and $[^3H]$thymidine incorporation, respectively. For proliferation, both INSβ and the control HEL peptides were presented on irradiated NOD splenocytes. Each bar represents the mean ± SD of triplicates. *G*, Illustrates production of IL-10 by agg Ig-INSβ-expanded and natural (Nil) $CD4^+CD25^+$ T cells in comparison with their $CD4^+CD25^-$ counterparts upon stimulation with plate-bound anti-CD3 Ab, as measured by ELISA. The bars represent the mean ± SD of triplicates. *H*, Agg Ig-INSβ-expanded splenic $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were coinjected with diabetic splenocytes into NOD.scid mice, and blood glucose levels were monitored weekly. A recipient group injected with diabetic splenocytes only (No transfer) was included as a control. Shown is the percentage of mice free of diabetes.

Agg Ig-INSβ suppresses T cell responses

IL-10 produced by the DC upon presentation of agg Ig-INSβ displays down-regulatory functions on the activation of specific T cells engaged to the DC through INSβ peptide. Indeed, when an

INSβ-specific Th1 cell line was incubated with DC and agg Ig-INSβ, the secretion of IFN-γ by the T cell line decreased as production of IL-10 by the DC increased (Fig. 2*A*). Such down-regulation of IFN-γ did not occur with sol Ig-INSβ, which did not induce IL-10 secretion by the DC (Fig. 2*B*). Neutralization of IL-10 during stimulation with agg Ig-INSβ restores IFN-γ production by the T cells (Fig. 2*C*).

In vivo, when NOD mice were given agg Ig-INSβ at a young age and then tested for T cell responses at a later time point, there was effective suppression of proliferation and IFN-γ production (Fig. 2, *D* and *E*). Untreated mice, whether diabetic or not, developed significant proliferation and IFN-γ production upon stimulation with INSβ, but not HEL peptide. Interestingly, agg Ig-INSβ-treated, but not untreated mice developed IL-10 responses upon stimulation with INSβ, but not HEL peptide (Fig. 2*F*). Overall, these findings indicate that agg Ig-INSβ induces tolerance of diabetogenic T cells most likely through IL-10 from APCs and/or Treg cells.

Agg Ig-INSβ delays diabetes in young NOD mice through IL-10-producing Treg cells

Agg Ig-INSβ was then tested for protection of young NOD mice against diabetes. Accordingly, animals were given agg Ig-INSβ at the preinsulitis stage (weeks 4, 5, and 6 of age), and the mice were monitored for blood glucose weekly up to week 26. As shown in Fig. 3*A*, agg Ig-INSβ delayed diabetes in all mice, except one up to week 20. Such delay remained significant by week 26, at which point only 30% of the mice had high blood glucose levels, while 80% of the untreated mice became diabetic. It is worth noting that agg Ig-HEL displayed a significant delay of diabetes up to week 16. Because Ig-HEL is made of the same Ig backbone (IgG2b isotype) as Ig-INSβ and upon aggregation cross-links FcγR on the presenting cells and induces IL-10 production by APCs, such a delay is most likely due to IL-10 bystander suppression. In fact, we have previously observed similar bystander suppression unrelated to Ag specificity with Ig-myelin chimeras (11–13). In contrast, sol Ig-INSβ, which does not induce IL-10 by APCs, was not as effective as agg Ig-INSβ in delaying the onset of diabetes (Fig. 3*B*). Although no animals were hyperglycemic by week 16 of age and some delay persisted until week 20, most of the mice became

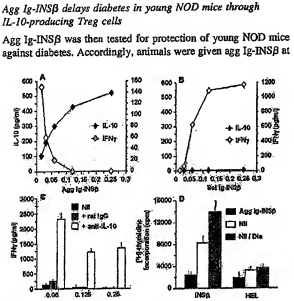

FIGURE 2. Agg Ig-INSβ down-regulates INSβ-specific T cells both in vitro and in vivo. Down-regulation of INSβ-specific Th1 cell line in vitro by agg (*A*) or sol Ig-INSβ (*B*) was assessed by measurement of IFN-γ using ELISA. IL-10 production by the presenting DC was also measured in the same culture well by ELISA. *C*, The effect of DC's IL-10 on IFN-γ secretion by the Th1 cell line was assessed by stimulation with graded amounts of agg Ig-INSβ in the presence of 40 μg of anti-IL-10 Ab or rat IgG control. Each bar represents the mean ± SD of triplicates. *D–F*, Mice were untreated (Nil) or given 300 μg of agg Ig-INSβ (Agg Ig-INSβ) at weeks 4, 5, and 6 of age, and their responses were analyzed on week 16. The analysis used purified splenic CD4 T cells that were stimulated with INSβ or HEL peptide presented on BSA-APCs. Splenic cells from untreated diabetic (Nil/dia) mice were included for comparison purposes. The proliferative responses (*D*) were measured by [^{3}H]thymidine incorporation, while IFN-γ (*E*) and IL-10 (*F*) production were assessed by ELISA.

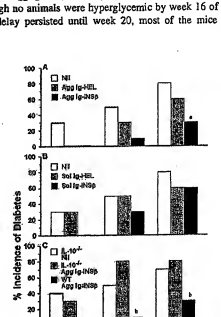

FIGURE 3. Expansion of CD4$^+$CD25$^+$ IL-10-producing T cells is required for effective suppression of diabetes in young NOD mice. Female NOD mice (10 per group) were given an i.p. injection of a saline solution containing 300 μg of either agg (*A*) or sol Ig-INSβ or Ig-HEL (*B*) at weeks 4, 5, and 6 of age, and then monitored for blood glucose levels weekly up to 26 wk of age. A group of mice that did not receive any injection (Nil) was included for control purposes. *C*, Groups (10 mice per group) of female wild-type (WT) and IL-10$^{-/-}$ NOD mice were given agg Ig-INSβ according to the same treatment regimen and monitored for blood glucose levels. A group of IL-10$^{-/-}$ mice that did not receive any treatment with agg Ig-INSβ was included for control purposes. *D*, Groups (10 mice per group) of mice were given agg Ig-INSβ according to the same treatment regimen, except that each injection was accompanied by 1 mg of anti-CD25 Ab or rat IgG control. A group of mice given anti-CD25 Ab without agg Ig-INSβ was included to serve as control. *a*, $p < 0.05$ compared with untreated mice; *b*, $p < 0.05$ compared with IL-10$^{-/-}$/agg Ig-INSβ-treated mice; *c*, $p < 0.05$ compared with untreated mice.

diabetic by week 26. Sol Ig-HEL did not display any significant delay of diabetes onset, indicating that the effect observed with Ig-INSβ is Ag specific. The role of IL-10 against diabetes at this young age became evident when agg Ig-INSβ was unable to delay the onset of diabetes in NOD mice deficient for IL-10 (Fig. 3*C*). Indeed, the incidence of diabetes was similar in agg Ig-INSβ-treated and untreated IL-10$^{-/-}$ NOD mice, but significantly higher than in the treated wild-type mice. Interestingly, when depleting anti-CD25 Ab accompanied the treatment, delay of disease did not occur (Fig. 3*D*). Indeed, the incidence of diabetes increased from 20 to 50% at weeks 20 and 30 to 70% at week 26 in animals treated with agg Ig-INSβ + rat IgG vs agg Ig-INSβ + anti-CD25 Ab. These results indicate that agg Ig-INSβ, which sustains IL-10 production from both APCs and Treg cells, down-regulates diabetogenic T cells and effectively protects young mice against diabetes.

Endogenous IL-10 opposes protection against diabetes upon treatment of IAA-positive mice with agg Ig-INSβ

Recently, it has been shown that IAA can be used as a marker for insulitis (26) and prediction of type 1 diabetes in young NOD mice (27). Similarly, among 58 female NOD mice that seroconverted to IAA production at the age of 8–11 wk, 84% had become diabetic by 30 wk of age, suggesting that our assay for detection of autoantibody is reliable and supports the notion that IAA can predict both diabetes (27) and most certainly insulitis (26). This offers a reference point to evaluate agg Ig-INSβ for reversal of diabetes at an early stage of the disease. Accordingly, NOD mice were given agg Ig-INSβ upon IAA seroconversion, as indicated, and monitored for blood glucose levels up to week 26 of age. Surprisingly, no significant delay of disease was observed, and the incidence of diabetes was similar in the mice treated with agg Ig-INSβ and Ig-HEL (Fig. 4*A*). The sol Ig-INSβ though showed some delay on week 20 relative to untreated or sol Ig-HEL-treated mice (Fig. 4*B*). Moreover, when a continuous treatment regimen was applied, a significant delay of the disease was observed with the sol, but not the agg form of Ig-INSβ (Fig. 4, *C* and *D*). Indeed, only 20% of sol Ig-INSβ-treated mice developed diabetes by week 20, and such a delay remained significant as only an additional 10% of mice became diabetic by 26 wk of age (Fig. 4*D*). The delay is Ag specific, as Ig-HEL had no significant delay or protection against diabetes at any time point and Ig-HEL-treated animals had a similar pattern of disease as the untreated mice. The disease pattern observed in agg Ig-INSβ-treated groups was also comparable to those seen with untreated or Ig-HEL-treated mice (Fig. 4*C*). Histological analysis at week 26 indicated that the mice treated continuously with sol Ig-INSβ and remaining free of diabetes had islet infiltration, but to a lesser extent than mice given sol Ig-HEL (Fig. 4*E*). The lack of efficacy of agg Ig-INSβ against diabetes was not due to irreversibility of the disease, but most likely to endogenous IL-10 induced by agg Ig-INSβ. This statement is supported by the observation that IAA-positive IL-10$^{-/-}$ mice reverse their diabetes upon treatment with agg Ig-INSβ, while the untreated mice do not (Fig. 4*F*). Indeed, the incidence of diabetes in these mice was 30% at week 26 of age, while the untreated animals had 70% incidence like wild-type NOD mice treated with agg Ig-INSβ. Overall, these results indicate that agg Ig-INSβ is not effective against diabetes upon IAA seroconversion most likely due to an undefined regulatory function of IL-10.

Agg Ig-INSβ stimulates rather than tolerizes islet-resident T cells

IL-10 has been ineffective against diabetes when expressed locally in β cells (5). Similarly, mobilization of IL-10 by agg Ig-INSβ is also ineffective against the disease after IAA seroconversion, a stage in which diabetogenic T cells would have migrated to the islets. One possible interpretation of these observations is that islet T cells are resistant to the modulatory function of IL-10. To test this premise, splenic (peripheral) and islet cells from diabetes-free 12-wk-old naive NOD mice were stimulated with agg Ig-INSβ and their IFN-γ responses were measured. Fig. 5 shows that agg Ig-INSβ reduced IFN-γ responses by the splenic cells, while the sol form of Ig-INSβ as well as free INSβ peptide did not (Fig. 5*A*). Addition of IL-10, however, reduced the response of the cells against free INSβ and sol Ig-INSβ to levels similar to those observed with agg Ig-INSβ. In contrast, agg Ig-INSβ stimulated significant IFN-γ responses by islet cells, while the sol form and free peptide did not (Fig. 5*B*). Interestingly, exogenous IL-10 boosts free INSβ and sol Ig-INSβ to support significant IFN-γ responses by the otherwise unresponsive islet cells. Neutralization of IL-10 with an anti-IL-10 Ab during stimulation with agg Ig-INSβ inhibits the IFN-γ responses by islet cells, while isotype-matched control Ab did not (Fig. 5*C*). Because islet infiltration includes CD8

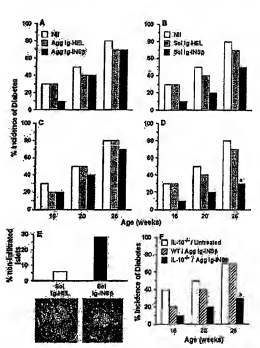

FIGURE 4. Treatment of diabetes in IAA-seropositive mice is much more effective when the regimen is devoid of IL-10. Mice (10 per group) that tested positive for IAA between the age of 8 and 11 wk were given an i.p. injection of 300 μg of agg (*A*) or sol (*B*) Ig-INSβ (■) or Ig-HEL (▨) on the week of seroconversion, as well as 7 and 14 days later. Other groups of mice were given a weekly injection of agg (*C*) or sol (*D*) Ig-INSβ (■) or Ig-HEL (▨) up to week 12. Subsequently, these mice received another 300 μg of Ig chimera every 2 wk until the age of 24 wk. This regimen is referred to as continuous treatment regimen. All mice were monitored for blood glucose from weeks 12 to 26 of age. An untreated group of mice (□) was included in all experiments for comparison purposes. *E*, Shows an *H&E* staining of islet sections and the percentage of noninfiltrated islets in the IAA-positive mice treated continuously with sol Ig-INSβ or Ig-HEL. The histological analyses illustrated in *E* were performed on nondiabetic mice at week 26 of age. *F*, Groups of IAA-positive IL-10$^{-/-}$ female NOD mice were subject to a continuous treatment regimen with agg Ig-INSβ (IL-10$^{-/-}$/Agg Ig-INSβ), and their incidence of diabetes is compared with untreated IL-10$^{-/-}$ (IL-10$^{-/-}$/Untreated) as well as wild-type NOD female mice treated with agg Ig-INSβ (WT/Agg Ig-INSβ). *a*, $p < 0.05$ compared with untreated mice; *b*, $p < 0.05$ compared with WT/agg Ig-INSβ-treated mice.

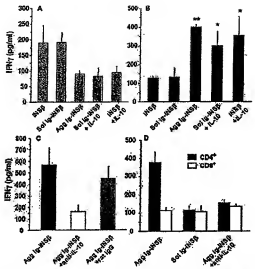

FIGURE 5. Islet INSβ-specific T cells develop IFN-γ responses upon stimulation with Ag in the presence of IL-10, while splenic T cells undergo down-regulation. Whole splenic (*A*) and islet (*B*) cells from 12-wk-old NOD female mice were stimulated with 18 μM INSβ, 1 μM agg, or sol Ig-INSβ in the absence or presence of 1 ng of rIL-10, as indicated, and their IFN-γ responses were measured. *C*, The stimulation of islet cells was conducted in the presence of 40 μg of anti-IL-10 Ab or isotype control rat IgG. *D*, Purified islet CD4 and CD8 T cells were incubated with BSA-APCs and 1 μM agg or sol Ig-INSβ with or without 40 μg/ml anti-IL-10 Ab. In all experiments, the incubation lasted 48 h, and cytokine measurement was done by ELISA using 100 μl of culture supernatant. Each bar represents the mean ± SD of triplicates after deduction of background levels obtained from cultures without Ag stimulation. These background levels were 3- to 8-fold lower than sol Ig-INSβ for the spleen (*A*) or agg Ig-INSβ for the islets cells (*B–D*). *, $p < 0.05$ compared with sol Ig-INSβ; **, $p < 0.01$ compared with sol Ig-INSβ.

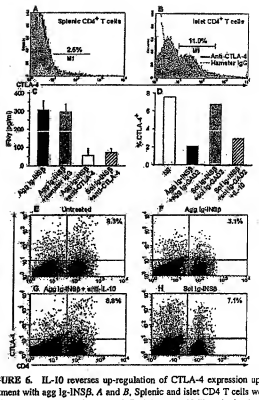

FIGURE 6. IL-10 reverses up-regulation of CTLA-4 expression upon treatment with agg Ig-INSβ. *A* and *B*, Splenic and islet CD4 T cells were purified by positive selection on anti-CD4 Ab-coated Miltenyi microbeads and stained with anti-CD4-FITC and PE-conjugated anti-CTLA-4 Ab or isotype control hamster IgG. The cells were gated on CD4 and analyzed for binding of anti-CTLA-4 or isotype control hamster IgG. The marker, M1, represents the cells positive for CTLA-4. *C*, Purified islet CD4 T cells were incubated with BSA-APCs and 1 μM agg or sol Ig-INSβ with or without 100 μg/ml anti-CTLA-4 Ab, then IFN-γ was measured by ELISA. The 4F10 Ab used here triggers rather than blocks the CTLA-4-inhibitory pathway. Each bar represents the mean ± SD of triplicates. *D*, The islet CD4 T cells were incubated with BSA-APCs and a 1 μM mixture of either agg or sol Ig-INSβ + Ig-GAD2 (1/1) in the presence or absence of 1 ng of rIL-10. The cells were then stained with anti-CD4-FITC and anti-CTLA-4-PE and analyzed as in *A* and *B*. For investigation of in vivo down-regulation of CTLA-4 by agg Ig-INSβ, IA^k-positive NOD female mice were untreated (*E*), given a three-injection regimen (as in Fig. 4*A*) of agg Ig-INSβ alone (*F*), agg Ig-INSβ accompanied by anti-IL-10 Ab (500 μg/injection), (*G*) or sol Ig-INSβ (*H*). Seven days later, the splenic CD4 T cells were purified and stained with anti-CD4 and anti-CTLA-4, as above.

among other T cells (28), the INSβ peptide contains a CD8 epitope (29, 30), and IL-10 has been shown to stimulate CD8 T cells (31), the IFN-γ responses obtained with islet cells could be due to cross-presentation of agg Ig-INSβ to CD8 T cells. Therefore, bulk islet cells were fractionated into CD4 and CD8 T cells, and stimulation with agg Ig-INSβ was reassayed. The results in Fig. 5*D* indicate that the postulate is incorrect, and CD4, but not the CD8 T cells were able to produce IFN-γ upon stimulation with agg Ig-INSβ. Furthermore, neutralization of IL-10 with an anti-IL-10 Ab inhibits IFN-γ responses by the CD4 T cells. These data indicate that islet and peripheral INSβ-specific CD4 T cells display differential susceptibility to IL-10.

Agg Ig-INSβ down-regulates CTLA-4 expression on islet T cells through endogenous IL-10

Upon migration to the islets, T cells would be exposed to Ag and undergo activation. Hypothetically, these cells would up-regulate CTLA-4 to deliver negative signals and control such activation (32, 33). IL-10 may down-regulate CTLA-4 to interfere with its inhibitory function and sustain activation of islet-resident T cells. Indeed, Fig. 6 shows that in the spleen of unmanipulated 12-wk-old mice, only 2.5% of CD4 T cells express surface CTLA-4 (Fig. 6*A*), while in the islets CTLA-4 expression was seen on 11% of the resident CD4 T cells (Fig. 6*B*). Interestingly, stimulation of the islet CD4 T cells with agg Ig-INSβ in the presence of anti-CTLA-4 Ab inhibited stimulation of IFN-γ production, while isotype control Ab did not (Fig. 6*C*). Moreover, anti-CTLA-4 Ab did not confer stimulatory function to sol Ig-INSβ, indicating that signaling through, rather than blockade of, CTLA-4 is the operative mechanism in this setting.

To test whether IL-10 interferes with expression of CTLA-4, islet CD4 T cells were stimulated with a mix (Ig-INSβ and Ig-GAD2) of Ig chimeras, and CTLA-4 expression was assessed. The addition of Ig-GAD2 together with Ig-INSβ in this assay is to maximize the number of specific CD4 T cells for analysis of CTLA-4 expression upon stimulation with Ag. Strikingly, the results depicted in Fig. 6*D* show that stimulation of islet T cells with agg chimeras significantly reduced the expression of CTLA-4. However, such a reduction did not occur with sol chimeras, but addition of IL-10 to the culture supported CTLA-4 down-regulation by the sol chimeras. In vivo, CTLA-4 expression on islet T cells was reduced from 8.3% in untreated mice to 3.1% in agg

Ig-INSβ-treated animals (Fig. 6, *E* and *F*). In fact, when tested for IFN-γ production, these cells showed higher levels of cytokine than untreated animals (248 pg/ml ± 46 vs 128 pg/ml ± 27). Moreover, coadministration of anti-IL-10 Ab with agg Ig-INSβ restored CTLA-4 expression, and the number of islet cells with significant surface CTLA-4 was similar to that observed in mice recipient of sol Ig-INSβ, which does not induce IL-10 production by APCs (Fig. 6, *G* and *H*). These results indicate that IL-10 produced by the APCs and/or Treg cells down-regulates CTLA-4 expression on islet-resident T cells.

Discussion

IL-10, an anti-Th1 cytokine and growth factor for Treg cells, prompted high expectations for modulation of autoreactive T cells and suppression of autoimmunity (14, 15, 17, 34, 35). Success has been achieved in a number of autoimmunity models, but IL-10 has shown variable results in type 1 diabetes (5–9). In this study, an approach for controlled mobilization of IL-10 was developed and used both in young insulitis-free and older IAA-positive mice to determine how the cytokine regulates diabetogenic CD4 T cells within and peripheral to the islets. It is shown that Ig-INSβ, an Ig expressing the diabetogenic INSβ peptide, can, upon aggregation, cross-link FcγRs and trigger the production of IL-10 by APCs (Fig. 1). In vitro, agg Ig-INSβ suppressed IFN-γ responses of INSβ-specific T cells, and such modulation was dependent upon IL-10 (Fig. 2). In vivo, young mice exposed to agg Ig-INSβ developed IL-10-producing Treg cells (Fig. 1), reduced their proliferative and IFN-γ responses (Fig. 2), and delayed their diabetes (Fig. 3). This protection against the disease was also IL-10 dependent as NOD mice deficient for the IL-10 gene were unable to delay their disease upon treatment with agg Ig-INSβ (Fig. 3). Moreover, depletion of IL-10-producing Treg cells abrogated agg Ig-INSβ-mediated protection against diabetes (Fig. 3). These observations suggest that endogenous IL-10, whether from APCs or Ag-expanded Treg cells, contributes significantly to the down-regulation of peripheral T cells in these young mice and sustains protection against the disease. IL-10 exercises anti-Th1 function through down-regulation of the expression of costimulatory molecules (31, 36). Our own investigation indicates that agg Ig-INSβ does not up-regulate B7 or CD40 on APCs (data not shown), which agrees with our previous reports showing that agg Ig-myelin chimeras made of the same Ig backbone as Ig-INSβ modulate T cells through lack of costimulation (11, 12). Thus, the mechanism we propose for protection against diabetes in the young mice suggests that IL-10 from the APCs and/or Treg cells most likely interferes with costimulation (see Fig. 7, *left panel*). This does not, however, exclude the possibility that Treg cells may be exercising additional suppressive function (37, 38) or that IL-10 may be directly affecting the diabetogenic T cells (39).

In contrast, this IL-10-driven protection against diabetes was not effective in older animals positive for IAA. Indeed, when agg Ig-INSβ was administered upon IAA seroconversion, protection was not achieved, despite the fact that the disease remains reversible and the sol form of Ig-INSβ delayed diabetes effectively (Fig. 4). Given the fact that in young animals most of the diabetogenic T cells remain peripheral to the islets, while in older mice a significant number of these cells would have become islet resident, we suspected that peripheral and islet-resident T cells display differential susceptibility to regulation by IL-10. This hypothesis proved correct, and splenic INSβ-specific T cells down-regulated IFN-γ production upon stimulation with agg Ig-INSβ, while islet T cells responded to such stimulation and produced significant amounts of IFN-γ (Fig. 5). However, sol Ig-INSβ, which does not induce IL-10 production by APCs, displayed opposite effects and stimulated IFN-γ responses by the splenic, but not islet T cells. IL-10 has previously been shown to stimulate CD8 T cells (31). Given the fact that INSβ encompasses a CTL epitope (29, 30), we thought that agg Ig-INSβ is cross-presented on MHC class I through the exogenous pathway and stimulates CD8 T cells that would be frequent in the islets during insulitis (40). Our prediction, however, proved incorrect, and upon separation of islet CD4 and CD8 T cells and stimulation with agg Ig-INSβ only the CD4 T cells responded and produced IFN-γ (Fig. 5*D*). Overall, these observations indicate that IL-10 is stimulatory for islet-resident diabetogenic CD4 T cells, but down-regulatory for the same cells when the encounter occurs peripheral to the islets.

Upon migration to the islets, T cells are presumably exposed to Ag and most likely undergo activation. CTLA-4 expression arises on activated T cells, providing a means to control excessive responses (32, 33). Thus, it is possible that upon IAA seroconversion, the islet-resident T cells up-regulate CTLA-4 expression. Upon treatment with agg Ig-INSβ, it may be that IL-10 interferes with CTLA-4-inhibitory function and stimulates T cell responses rather than tolerance. This postulate proved correct, and islet, but

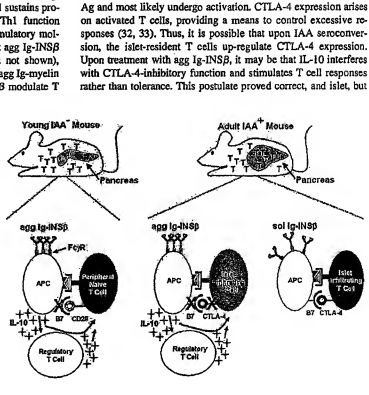

FIGURE 7. Proposed model for IL-10 regulation of peripheral and islet-resident diabetogenic T cells.

not splenic T cells from the same animal displayed up-regulated expression of surface CTLA-4 (Fig. 6, *A* and *B*). Interestingly, addition of anti-CTLA-4 Ab during incubation with agg Ig-INSβ restored the inhibitory function of CTLA-4 and the T cells were not able to produce IFN-γ (Fig. 6*C*). Moreover, stimulation of islet T cells with agg Ig chimeras reduced the surface expression of CTLA-4, while stimulation with sol chimeras did not, unless supplemented with exogenous IL-10 (Fig. 6*D*). In vivo, treatment of IAA-positive mice with agg Ig-INSβ down-regulated CTLA-4 expression on islet T cells (Fig. 6, *E* and *F*). However, neutralization of IL-10 during administration of agg Ig-INSβ restored CTLA-4 expression (Fig. 6*G*). Thus, IL-10 sustains stimulation of previously activated islet-resident T cells by down-regulation of CTLA-4 expression and interference with its inhibitory function. In fact, administration of anti-CTLA-4 upon IAA seroconversion completely abrogated the onset of diabetes (data not shown). Therefore, interruption of CTLA-4-inhibitory function by IL-10 promotes activation rather than tolerance. The *median panel* of Fig. 7 proposes that IL-10 down-regulates both costimulatory molecules and CTLA-4, resulting in loss of inhibitory control of diabetogenic T cells. This will ultimately sustain stimulation, as previously activated lymphocytes do not require costimulation (41, 42). The fact that sol Ig-INSβ, not inducing IL-10, was able to delay disease at this stage bodes well with the findings. The *right panel* of Fig. 7 proposes that sol Ig-INSβ does not sustain activation of the cells because the APC at this inflammatory site express costimulatory molecules that should engage CTLA-4, which is not down-regulated by the sol Ig-INSβ (Fig. 6*D*). The end result then is inhibition of T cell activation and delay of diabetes.

Overall, agg Ig-INSβ tolerizes T cells in the periphery and limits input into the islets, thus effectively suppressing the disease when given at a young age before insulitis. Upon IAA seroconversion, agg Ig-INSβ will exercise down-regulation of peripheral T cells, limiting the seeding of islets by naive T cells, but will compensate for the shortage by stimulating and sustaining vigorous activation of islet-resident cells that have migrated before the treatment or have escaped peripheral tolerance. Sol Ig-INSβ is less effective in tolerizing peripheral T cells due to the lack of IL-10. However, upon IAA seroconversion, sol Ig-INSβ will compensate for the moderate tolerance in the periphery by not sustaining activation of islet-resident T cells. This mechanism will require continual treatment and show reduced infiltration. This model agrees with the report showing that anti-CTLA-4 Ab delays passive diabetes induced by transfer of activated pathogenic T cells (43). The findings are also in good standing with observations indicating that local expression of IL-10 exacerbates the disease (5, 7) and delivery of IL-10 at an older age is not effective against diabetes (9). Thus, the model reconciles the variable functions associated with IL-10 (6). The notion that encounter of the T cells with IL-10 before migration to the islets has a different outcome from encounters that happen within the islets is also supported by studies demonstrating that delivery of IL-10 at a young age (before insulitis) delays diabetes, while it is ineffective against disease in older animals with progressive insulitis (9).

Another point we emphasize is that this interplay between IL-10 and CTLA-4 may contribute to the development of spontaneous diabetes. Treg cells develop in the normal T cell repertoire and are presumed to sustain peripheral tolerance (37, 38). An initial exposure of β cell-associated self Ags would activate diabetogenic T cells, but could also expand Treg cells to control pathogenicity (44). However, if those Treg cells produce IL-10, an interplay with CTLA-4 would be put into motion and their function would be rather counterproductive, resulting in sustained T cell activation and exacerbation of diabetes. This possibility, however, remains to be investigated. Recently, we found that decline of membrane-bound TGFβ can also nullify the suppressive function of Tregs, leading to development of diabetes (45).

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References

1. Castano, L., and G. S. Eisenbarth. 1990. Type-1 diabetes: a chronic autoimmune disease of human, mouse, and rat. *Annu. Rev. Immunol. 8:647.*
2. Tisch, R., and H. O. McDevitt. 1996. Insulin dependent diabetes mellitus. *Cell 85:291.*
3. Delovitch, T., and B. Singh. 1997. The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity 7:727.*
4. Andre, I., A. Gonzalez, B. Wang, J. Katz, C. Benoist, and D. Mathis. 1996. Checkpoints in the progression of autoimmune disease: lessons from diabetes models. *Proc. Natl. Acad. Sci. USA 93:2260.*
5. Wogensen, L., M.-S. Lee, and N. Sarvetnick. 1994. Production of interleukin 10 by islet cells accelerates immune-mediated destruction of β cells in nonobese diabetic mice. *J. Exp. Med. 179:1379.*
6. Balasa, B., and N. Sarvetnick. 1996. The paradoxical effects of interleukin 10 in the immunoregulation of autoimmune diabetes. *J. Autoimmun. 9:283.*
7. Balasa, B., A. La Cava, K. Van Gunst, L. Mocnik, D. Balakrishna, N. Nguen, L. Tucker, and N. Sarvetnick. 2000. A mechanism for IL-10-mediated diabetes in the nonobese diabetic (NOD) mouse: ICAM-1 deficiency blocks accelerated diabetes. *J. Immunol. 165:7230.*
8. Phillips, J. M., N. M. Parish, M. Drage, and A. Cooke. 2001. Cutting edge: interactions through the IL-10 receptor regulate autoimmune diabetes. *J. Immunol. 167:6087.*
9. Yang, Z., M. Chen, R. Wu, L. B. Fialkow, J. S. Bromber, M. McDuffie, A. Naji, and J. Nadler. 2002. Suppression of autoimmune diabetes by viral IL-10 gene transfer. *J. Immunol. 168:6479.*
10. Legge, K. L., B. Min, N. T. Potter, and H. Zaghouani. 1997. Presentation of a T cell receptor antagonist peptide by immunoglobulins ablates activation of T cells by a synthetic peptide or proteins requiring endocytic processing. *J. Exp. Med. 185:1063.*
11. Legge, K. L., B. Min, J. J. Bell, J. C. Caprio, L. Li, R. K. Gregg, and H. Zaghouani. 2000. Coupling of peripheral tolerance to endogenous interleukin 10 promotes effective modulation of myelin-activated T cells and ameliorates experimental allergic encephalomyelitis. *J. Exp. Med. 191:2039.*
12. Legge, K. L., J. J. Bell, L. Li, R. K. Gregg, J. C. Caprio, and H. Zaghouani. 2001. Multi-modal antigen specific therapy for autoimmunity. *Int. Rev. Immunol. 20:593.*
13. Legge, K. L., R. K. Gregg, R. Maldonado-Lopez, L Li, J. C. Caprio, M. Moser, and H. Zaghouani. 2002. On the role of dendritic cells in peripheral T cell tolerance and modulation of autoimmunity. *J. Exp. Med. 196:217.*
14. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, J. de Vries, and M.-G. Roncarolo. 1997. Generation of a novel regulatory CD4+ T-cell population, which inhibits antigen-specific T-cell responses. *Nature 389:737.*
15. Assemas, C., S. Meuze, M. W. Leach, R. L. Coffman, and F. Powrie. 1999. An essential role for IL-10 in the function of Treg cells that inhibit intestinal inflammation. *J. Exp. Med. 190:995.*
16. Barral, F. J., D. J. Cua, A. Boonstra, D. F. Richards, C. Crain, H. F. Savelkoul, R. de Waal-Malefyt, R. L. Coffman, C. M. Hawrylowicz, and A. O'Garra. 2002. In vitro generation of interleukin 10-producing regulatory CD4+ T cells is induced by immunosuppressive drugs and inhibited by T helper (Th)1- and Th2-inducing cytokines. *J. Exp. Med. 195:603.*
17. Sundstedt, A., E. J. O'Neill, K. S. Nicolson, and D. C. Wraith. 2003. Role for IL-10 in suppression mediated by peptide-induced Treg cells in vivo. *J. Immunol. 170:1240.*
18. Daniel, D., and D. R. Wegmann. 1996. Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B (9-23). *Proc. Natl. Acad. Sci. USA 93:956.*
19. Heath, V. L., P. Hutchings, D. J. Fewell, A. Cooke, and D. Mason. 1999. Peptides derived from murine insulin are diabetogenic in both rats and mice, but the disease-inducing epitopes are different: evidence against a common environmental cross-reactivity in the pathogenicity of diabetes. *Diabetes 48:2157.*
20. Serreze, D. V., H. D. Chapman, C. M Post, E. A. Johnson, W. L. Suarez-Pinzon, and A. Rabinovitch. 2001. Th1 to Th2 cytokine shifts in nonobese diabetic mice: sometimes an outcome, rather than the cause of diabetes resistance elicited by immunostimulation. *J. Immunol. 166:1352.*
21. Chao, C.-C., H.-K. Sytwu, E. L. Chen, J. Toma, and H. O. McDevitt. 1999. The role of MHC class II molecules in susceptibility to type 1 diabetes: identification of peptide epitopes and characterization of the T cell repertoire. *Proc. Natl. Acad. Sci. USA 96:9299.*
22. Latek, R. R., A. Suri, S. J. Petzold, C. A. Nelson, O. Kanagawa, E. R. Unanue, and D. H. Fremont. 2000. Structural basis of peptide binding and presentation by the diabetes-associated MHC class II molecule of NOD mice. *Immunity 12:699.*

23. Faveeuw, C., M. C. Gagnerault, and F. Lepault. 1995. Isolation of leukocytes infiltrating the islets of Langerhans of diabetes-prone mice for flow cytometric analysis. *J. Immunol. Methods 187:163.*
24. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of $CD4^+CD25^+$Treg cells. *Nat. Immunol. 4:330.*
25. Li, L., H.-H. Lee, J. J. Bell, R. K. Gregg, J. S. Ellis, A. Gessner, and H. Zaghouani. 2004. IL-4 utilizes an alternative receptor to drive apoptosis of Th1 cells and skews neonatal immunity towards Th2. *Immunity 20:429.*
26. Robles, D. T., G. S. Eisenbarth, N. J. M. Dailey, L. B. Peterson, and L. Wicker. 2003. Insulin autoantibodies are associated with islet inflammation but not always related to diabetes progression in NOD congenic mice. *Diabetes 52:882.*
27. Yu, L., D. T. Robles, N. Abiru, P. Kaur, M. Rewers, K. Kelemen, and G. S. Eisenbarth. 2000. Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes. *Proc. Natl. Acad. Sci. USA 97:1701.*
28. Ablamunits, V., D. Elias, and I. R. Cohen. 1999. The pathogenicity of islet-infiltrating lymphocytes in the non-obese diabetic (NOD) mouse. *Clin. Exp. Immunol. 115:260.*
29. Anderson, B., B.-J. Park, J. Verdaguer, A. Abrani, and P. Santamaria. 1999. Prevalent $CD8^+$ T cell response against one peptide/MHC complex in autoimmune diabetes. *Proc. Natl. Acad. Sci. USA 96:9311.*
30. Wong, F. S., A. K. Moustakas, L. Wen, G. K. Papadopoulos, and C. A. Janeway, Jr. 2002. Analysis of structure and function relationships of an autoantigenic peptide of insulin bound to H-2K(d) that stimulates CD8 T cells in insulin-dependent diabetes mellitus. *Proc. Natl. Acad. Sci. USA 99:5551.*
31. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol. 19:683.*
32. Chambers, C. A., M. S. Kuhns, J. G. Egen, and J. P. Allison. 2001. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu. Rev. Immunol. 19:565.*
33. Salomon, B., and J. A. Bluestone. 2001. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu. Rev. Immunol. 19:225.*
34. Zheng, X., A. Steele, W. Hancock, A. C. Stevens, P. W. Nickerson, P. Roy-Chaudhury, Y. Tian, and T. B. Strom. 1997. A noncytolytic IL-10/Fc fusion protein prevents diabetes, blocks autoimmunity, and promotes suppressor phenomena in NOD mice. *J. Immunol. 158:4507.*
35. Moritani, M., K. Yoshimoto, S. Ii, M. Kondo, H. Iwahana, T. Yamaoka, T. Sano, N. Nakano, H. Kikutani, and M. Itakura. 1996. Prevention of adoptively transferred diabetes in nonobese diabetic mice with IL-10-transduced islet-specific Th1 lymphocytes. *J. Clin. Invest. 98:1851.*
36. Ding, L., P. S. Linsley, L.-Y. Huang, R. N. Germain, and E. M. Shevach. 1993. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J. Immunol. 151:1224.*
37. Sakaguchi, S. 2000. Treg cells: key controller of immunologic self tolerance. *Cell 101:455.*
38. Shevach, E. M. 2000. Treg cell in autoimmunity. *Annu. Rev. Immunol. 18:423.*
39. Joss, A., M. Akdis, A. Faith, K. Blaser, and C. A. Akdis. 2000. IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway. *Eur. J. Immunol. 30:1683.*
40. Wegmann, D. R., M. Norbury-Glaser, and D. Daniel. 1994. Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice. *Eur. J. Immunol. 24:853.*
41. McKnight, A. J., V. L. Perez, C. M. Shea, G. S. Gray, and A. K. Abbas. 1994. Costimulator dependence of lymphokine secretion by naive and activated $CD4^+$ T lymphocytes from TCR transgenic mice. *J. Immunol. 152:5220.*
42. Schweitzer, A. N., and A. H. Sharpe. 1998. Studies using antigen-presenting cells lacking expression of both B7-1 (CD80) and B7-2 (CD86) show distinct requirements for B7 molecules during priming versus restimulation of Th2 but not Th1 cytokine production. *J. Immunol. 161:2762.*
43. Luhder, F., C. Chambers, J. P. Allison, C. Benoist, and D. Mathis. 2000. Pinpointing when T cell costimulatory receptor CTLA-4 must be engaged to dampen diabetogenic T cells. *Proc. Natl. Acad. Sci. USA 97:12204.*
44. Szanya, V., J. Ermann, C. Taylor, C. Holness, and C. G. Fathman. 2002. The subpopulation of $CD4^+CD25^+$ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. *J. Immunol. 169:2461.*
45. Gregg, R. K., R. Jain, S. J. Schoenleber, R. Divekar, J. J. Bell, H.-H. Lee, P. Yu, and H. Zaghouani. 2004. A sudden decline in active membrane-bound TGFβ impairs both Treg function and protection against autoimmune diabetes. *J. Immunol. 173:7803.*